

Cyk-4 polypeptides, DNA molecules encoding them and  
their use in screening methods

The present application claims the benefit, under 35  
5 U.S.C. § 119, of the earlier filing dates of European  
Patent Application No. EP 00 112 880.0, filed June 19,  
2000; European Patent Application No. EP 01 110 554.1,  
filed April 30, 2001; U.S. Provisional Application No.  
60/241,231, filed October 18, 2000; and U.S.  
10 Provisional Application, entitled Cyk-4 polypeptides,  
DNA molecules encoding them and their use in screening  
methods, Atty. Docket No. 0652.2260000, filed June 13,  
2001. The contents of each of these applications are  
entirely incorporated herein by reference.  
15 The present invention relates to a new protein Cyk-4,  
which is involved in cytokinesis, to its use in  
screening assays, and to therapies interfering with  
cell division, in particular tumor therapy.

The process of cytokinesis produces two daughter cells  
20 from a single parental cell and permanently segregates  
the products of the cell division cycle. Cytokinesis  
is one of the few processes in biology known to require  
coordination between microtubules and actin filaments.  
Indeed, in animal cells there are two steps in  
25 cytokinesis that rely on interactions between  
microtubules and the actin-based contractile ring (for  
review see (Field et al., 1999; Glotzer, 1997). In the  
first instance, the microtubule-based mitotic spindle  
specifies the position of the contractile ring. This

allows the division plane to be positioned so that the separated chromosomes are partitioned equally into the two daughter cells. Subsequently, after ingress of the cleavage furrow, there is a second step that

5 depends on both microtubules and the contractile ring. Completion of cytokinesis requires the central spindle, which contains bundled, antiparallel microtubules. The molecular mechanisms underlying these two microtubule dependent steps in cytokinesis are not known.

10 The degree to which the progression of cytokinesis depends on the central spindle varies somewhat in different experimental organisms. In invertebrate embryos, a transient interaction between astral microtubules of the mitotic spindle and the cell cortex

15 is sufficient to position the cleavage furrow (Rappaport, 1985). Furrows specified in this manner ingress, but if the spindle is removed, these furrows do not usually complete cytokinesis (Rappaport, 1985). In contrast, in *Drosophila* spermatocytes, contractile

20 ring formation requires the central spindle (Giansanti et al., 1998). Similarly, in cultured mammalian cells, astral microtubules appear to be insufficient to induce furrow ingress, instead the presence or absence of a central spindle determines whether or not a cleavage

25 furrow forms (Cao and Wang, 1996; Eckley et al., 1997; Rieder et al., 1997; Savoian et al., 1999; Wheatley and Wang, 1996). Moreover, in cultured cells, the central spindle is also required for completion of cytokinesis (Wheatley and Wang, 1996). In *C. elegans* embryos, as in

30 invertebrate embryos, only the later stages of cytokinesis appear to depend on the central spindle. Embryos depleted of the kinesin-like protein

ZEN-4/CeMKlp1 fail to assemble the central spindle, yet cleavage furrows form and ingress, but cytokinesis does not proceed to completion (Powers et al., 1998; Raich et al., 1998). In summary, the initiation of 5 cytokinesis depends on the central spindle in some but not in all organisms, whereas there appears to be a general requirement for the central spindle for the completion of cytokinesis in animal cells. While it is clear that the central spindle plays an important role 10 in cytokinesis the underlying mechanism remains elusive.

Cleavage furrow ingress is driven by the actin-based contractile ring. Like many actin-based structures, the contractile ring requires the RhoA GTPase for its 15 assembly. Rho family GTPases are thought to act as molecular switches that cycle between inactive GDP-bound forms and active GTP-bound forms; their ability to exchange and hydrolyze GTP is regulated by additional factors, the so-called guanine nucleotide 20 exchange factors (GEFs) and GTPase activating proteins (GAPs). Inactivation of RhoA by the exoenzyme C3 (Aktories and Hall, 1989), inhibits cytokinesis in a wide variety of experimental settings by causing disassembly of cortical actin structures and the 25 contractile ring (Drechsel et al., 1997; Kishi et al., 1993; Mabuchi et al., 1993; Moorman et al., 1996; O'Connell et al., 1999). Further, a Rho GEF is essential for cytokinesis (Prokopenko et al., 1999; Tatsumoto et al., 1999). GTP-bound RhoA interacts with 30 a number of putative effectors including formins, Rho Kinase, Citron kinase, and a regulatory subunit of myosin phosphatase (for review see (Van Aelst and

D'Souza-Schorey, 1997). The requirement for RhoA in cytokinesis may reflect its ability to regulate formins since members of the formin gene family are required for cytokinesis in budding yeast (BN11/BNR1) (Imamura et al., 1997), fission yeast (Cdc12) (Chang et al., 1997), *Drosophila* (*dia*) (Castrillon and Wasserman, 1994) and *C. elegans* (*cyk-1*) (Swan et al., 1998). Several formins also bind to profilin (Chang et al., 1997 ; Evangelista et al., 1997; Imamura et al., 1997; Watanabe et al., 1997), a key regulator of actin polymerization. It is conceivable that GTP-bound RhoA promotes contractile ring assembly by activating actin polymerization via the formins and by activating myosin motor activity.

15 There is compelling evidence that the microtubule-based central spindle and the actin-based cleavage furrow are both essential for cytokinesis. Next, the question was asked how these two cytoskeletal polymers interact. There are a few cases in which an interaction between

20 the microtubule and actin cytoskeletal systems have been characterized. Examples include nuclear positioning in budding yeast (Carminati and Stearns, 1997; Fujiwara et al., 1999; Miller et al., 1999), spindle orientation in epithelial cells (Busson et al., 1998) and in certain asymmetrically dividing cells, such as the posterior blastomere of the two cell *C. elegans* embryo (Gönczy et al., 1999a; Hyman and White, 1987; Skop and White, 1998; Waddle et al., 1994). In these examples there is evidence that the

25 dynein/dynactin microtubule motor complex may mediate the interaction of microtubules with the cell cortex.

It was an object of the invention to elucidate the microtubule dependent steps in cytokinesis. In particular, it was sought to define the role of the central spindle in this process, in order to be able to 5 interfere with this process and thus with cytokinesis, which provides a new approach for therapy, in particular cancer therapy.

Therefore, to solve the problem underlying the invention, it was investigated how the central spindle 10 assembles and how it functions in cytokinesis.

In the present invention, a novel gene designated *cyk-4* was identified and cloned and its role in the early divisions of the *C. elegans* embryo was characterized. It was shown that *Cyk-4* is required for the late stages 15 of cytokinesis. Interestingly, *cyk-4* mutant embryos fail to assemble the central spindle. Positional cloning and localization studies revealed that the *cyk-4* gene encodes a novel GTPase activating protein (GAP) for the Rho family of GTPases that localizes to the 20 central spindle. The missense mutation in the *cyk-4* (*t1689ts*) allele is found in a domain dispensable for GAP activity suggesting that *Cyk-4* may have another function in addition to activating GTP hydrolysis by Rho family proteins. Accordingly, it was found that 25 *Cyk-4* and the kinesin-like protein *Zen-4/CeMKlp1* are interdependent for their proper localization. Based on these data, a model is proposed by which *Cyk-4* acts in concert with *Zen-4/CeMKlp1* to assemble the central spindle. The concentration of *Cyk-4* to the central 30 spindle would then serve to target the GAP domain to the fully ingressed contractile ring where it could

promote GTP hydrolysis by RhoA, thereby facilitating the completion of cytokinesis.

In further experiments, the biochemical state of CYK-4 and ZEN-4 has been examined and it was found that they 5 do indeed associate in vivo. These proteins also efficiently associate in vitro and an in vitro binding assay has been used to molecularly dissect the regions of both proteins that are necessary and sufficient for this interaction. It is shown that the protein encoded 10 by the ts allele *cyk-4(t1689)* is defective in binding to ZEN-4. The identification of mutations in *zen-4* that partially rescue *cyk-4(t1689)* and map to the CYK-4 binding region provides strong evidence for the importance of the interaction between CYK-4 and ZEN-4 15 for progression of cytokinesis. It has also been shown that the human orthologs of CYK-4 and ZEN-4, HsCYK-4/MgcRacGAP and MKLP1, are in a complex which has been purified. The complex contains approximately stoichiometric amounts of HsCYK-4 and MKLP1. The human 20 complex localizes to the central spindle and to division remnants, as do the nematode proteins. Thus, in the present invention, an evolutionarily conserved complex that is essential for central spindle assembly has been identified.

25 The division of a cell into two daughters requires dynamic interactions between the microtubule-based mitotic spindle and the actin-based contractile ring. In animal cells the position of the cleavage furrow, an actomyosin-based structure, is determined by the

mitotic spindle in a manner that is poorly understood. In recent years is has become clear that the central spindle, an array of microtubule bundles that forms during anaphase, also plays an important role in 5 cytokinesis. To gain insight into this fundamental cellular process, the cytokinesis-defective mutant, cyk-4, was analyzed. Cytological analysis reveals that cyk-4 mutant embryos fail to assemble the central spindle. Though lacking a central spindle, cyk-4 10 mutant embryos furrow extensively, but they fail to complete cytokinesis. The mutation responsible for the cyk-4 phenotype was found to be a missense mutation in a gene encoding a Rho family GAP that, *in vitro*, stimulates GTP hydrolysis by Rho, Rac, and Cdc42. CYK-4 15 localizes to the central spindle and to cell division remnants. CYK-4 colocalizes with the ZEN-4/CeMKLP1 kinesin-like protein. Moreover, CYK-4 and ZEN-4/CeMKLP1 are interdependent for their localization. It is concluded that the CYK-4 GAP and the ZEN-4/CeMKLP1 20 kinesin-like protein cooperate to assemble the central spindle. Furthermore, it is proposed that a concentrated source of CYK-4 GAP on the central spindle could downregulate the RhoA GTPase and thereby promote the late stages of cytokinesis. The findings of the 25 present invention provide a model for CYK-4 dependent assembly of the central spindle:

In *cyk-4* mutant embryos the robust microtubule bundles that constitute the central spindle do not form. Instead, the spindle develops into two mitotic asters 30 separated by a few overlapping, disorganized,

microtubules. A similar phenotype is observed in *zen-4* mutant embryos (Powers et al., 1998; Raich et al., 1998). Thus both the ZEN-4/CeMKLP1 kinesin-like protein and the CYK-4 GAP are essential for this microtubule  
5 bundling. The *Drosophila* orthologue of ZEN-4/CeMKLP1 is also required for cytokinesis, though it seems to be required for all aspects of furrow ingressoin (Adams et al., 1998). Members of the MKLP1 subfamily of kinesin-like proteins have microtubule bundling activity *in*  
10 *vitro* (Kuriyama et al., 1994; Nislow et al., 1992). However, *in vivo*, ZEN-4 mediated microtubule bundling requires CYK-4.

In answering the question how CYK-4 and ZEN-4 could cooperate to assemble the central spindle, it is  
15 proposed that a complex containing multiple motor proteins could specifically localize to overlapping, antiparallel microtubules (figure 10). If such a motor complex transits along a microtubule it might continue to an end and dissociate. However, if such a motor  
20 complex transited along a microtubule in the vicinity of an antiparallel microtubule, the complex might bind simultaneously to both microtubules and attempt to move alternately in opposite directions, the net result being that the complex would concentrate in the region  
25 of microtubule overlap. Since CYK-4 does not have a microtubule motor domain, yet it is essential for the formation of the central spindle, it is proposed that CYK-4 forms a complex with multiple ZEN-4 homodimers that localizes to and stabilizes overlapping  
30 antiparallel microtubules (figure 10).

It was of interest to find out if the RhoGAP activity of CYK-4 necessary to promote microtubule bundling by ZEN-4. It is proposed that central spindle assembly is unlikely to require CYK-4 GAP activity. This is 5 suggested by two lines of evidence. First, central spindle assembly is defective in the *cyk-4(t1689ts)* allele that carries a missense mutation at amino acid 15. This substitution is distant from the C-terminal GAP domain, and *in vitro*, the amino terminus 10 of CYK-4 is dispensable for GAP activity. Thus this allele would be predicted to retain catalytic activity *in vivo* and therefore GAP activity is not sufficient for central spindle assembly. Moreover, Rho RNAi experiments reveal that central spindle assembly is Rho 15 independent, suggesting that Rho GAP activity is not required for this process. Thus CYK-4 may act to promote central spindle assembly, independent of its GAP activity.

In the present invention, the function of the CYK-4 gap 20 domain was analysed:

If CYK-4 function in central spindle assembly is independent of the Rho GTPase, it was of interest to determine the function of the CYK-4 GAP domain. CYK-4 is likely bifunctional, one function being to promote 25 the assembly of the central spindle, the second function being to promote GTP hydrolysis by Rho family members. These two functions might be related in that the first function would serve to concentrate CYK-4 at a site where GAP activity is required. It may be 30 assumed that CYK-4 GAP activity is required late in cytokinesis, to promote GTP hydrolysis by a Rho family

GTPase whose downregulation causes disassembly of the contractile ring and cell separation (fig. 10).

It was determined which GTPase might CYK-4 act on to promote cytokinesis. The CYK-4 GAP domain has all the 5 hallmarks of a Rho family GAP and it may therefore be expected that it will act on this subfamily of the GTPase superfamily. Like many other RhoGAPs, the GAP domain of CYK-4 is promiscuous in its ability to promote GTP hydrolysis on Rho, Rac, and Cdc42 (Lamarche 10 and Hall, 1994). The strongest piece of evidence that CYK-4 acts on Rho is based on the observation that of the GTPases tested, Rho is the only one that is clearly essential for cytokinesis. The requirement for Rho in cell division is well documented in a variety of 15 experimental systems. To date there is no evidence that Rac is required for cytokinesis and the data using RNAi to deplete Rac also failed to detect a role for this GTPase in this process. Moreover, it has been recently reported that *ced-10* mutants, which are defective in 20 corpse engulfment subsequent to apoptosis and distal tip cell migration, contain mutations in the *rac* gene (Reddien and Horvitz, 2000). *ced-10* mutants do not have any gross phenotypes indicative of a role in cytokinesis. Moreover, *Rac1* deficient mice are 25 gastrulation defective, but the embryos do not contain multinucleate cells indicative of a cell division defect (Sugihara et al., 1998). The sum of these data argue that Rac is not an essential target of CYK-4 during cytokinesis. With regard to Cdc42, previous 30 studies have implicated this GTPase in cytokinesis (Drechsel et al., 1997; Dutartre et al., 1996). Superficially, the weakly penetrant cytokinesis

phenotype observed in *Cdc42(RNAi)* embryos is consistent with these earlier data. However the *Cdc42(RNAi)* embryos that are cytokinesis defective are also osmotically swollen and therefore cytokinesis defect 5 may be indirect. Thus at this juncture it appears most likely that RhoA is the key substrate for the CYK-4 GAP activity.

Further support for the hypothesis that completion of cytokinesis requires downregulation of RhoA by CYK-4 10 would be supported by experiments in which the *cyk-4* phenotype is phenocopied by RhoA mutants that are hydrolysis defective. However the genetic tools necessary to express such dominant mutants in the early *C. elegans* embryo are currently unavailable. It is 15 surprising that the CYK-4 GAP domain is less active towards RhoA as compared to Rac or Cdc42, if indeed RhoA is the relevant target of its GAP activity. One possible explanation is that full length CYK-4 has a different activity profile as compared to the isolated 20 GAP domain. A more interesting possibility is that CYK-4 localization is important for CYK-4 GAP activity. The phenotype of *cyk-4* mutant embryos suggests that CYK-4 needs to act when the contractile ring is in close proximity to the central spindle. Since CYK-4 is 25 concentrated on the central spindle at this time, its high local concentration might overcome its lower activity towards RhoA.

In the present invention, the central spindle was shown to be at the center of cytokinesis:

There appear to be at least two microtubule-dependent steps in cytokinesis, contractile ring positioning and 5 completion of cytokinesis. In some cells both processes are dependent on the central spindle. An important open question is whether these two reactions are mechanistically similar. While assembly of the contractile ring requires activation of RhoA, it was 10 previously shown that the position of the contractile ring is specified in a RhoA-independent manner in *Xenopus* embryos (Drechsel et al., 1997). It has been shown here that a Rho GAP is required for the late stages of cytokinesis, suggesting that the second 15 process, completion of cytokinesis, does involve RhoA. It is therefore assumed that the two microtubule-dependent steps in cytokinesis are distinct.

CYK-4 and ZEN-4 are not the only components of the central spindle, a number of other components, some of 20 which are required for cytokinesis, are also present at this site. Polo kinase is known to associate with MKLP1 and to concentrate in the central spindle (Adams et al., 1998; Lee et al., 1995), and this kinase is essential for cytokinesis. Rho associated kinase also 25 localizes to this site (Kosako et al., 1999). The AIR-2 aurora-like kinase localizes to the central spindle (Schumacher et al., 1998). This kinase seems to be required primarily for chromosome segregation (Woppard and Hodgkin, 1999), its direct involvement in 30 cytokinesis requires further analysis. INCENP and the TD-60 antigen also localize to the central spindle and

there is evidence that they may play a role in cytokinesis (Eckley et al., 1997; Mackay et al., 1998; Martineau-Thuillier et al., 1998; Savoian et al., 1999). Interestingly, a Rho GEF that is required for 5 cytokinesis, ECT2, also accumulates on the central spindle (Tatsumoto et al., 1999), however the *Drosophila* ortholog, Pebble, does not localize in this manner (Prokopenko et al., 1999).

Further analysis of the specific functions of all of 10 these cytokinesis regulators is the basis to determine which of these proteins are functionally interdependent as has been shown is the case for Cyk-4 and Zen-4/CeMKlp1.

Furthermore, it was shown that the central spindle is a 15 structure that is essential for completion of cytokinesis. Two proteins required for central spindle assembly, CYK-4 and ZEN-4, were shown to co-localize to the central spindle and to somehow act in concert with one another. In experiments of the present invention, 20 it was demonstrated that *in vivo*, CYK-4 and ZEN-4 are present in an evolutionarily conserved protein complex and the nature of this complex in *C. elegans* embryos and in human cells was characterized in detail. These data suggest that the CYK-4/ZEN-4 complex, which was 25 named centalspindlin, consists of a two molecules each of CYK-4 and ZEN-4. CYK-4 binds to the neck region of the ZEN-4 kinesin, raising the possibility that the motor activity of the ZEN-4 is directly regulated by the CYK-4 RhoGAP.

Analytical biochemistry of the native centalspindlin complex isolated from *C. elegans* embryos demonstrates the existence of a complex containing the ZEN-4 kinesin and the CYK-4 RhoGAP. In vitro binding experiments were 5 used to define the critical determinants for this interaction and to demonstrate that the two subunits are able to self associate. Genetic and biochemical suppression of the CYK-4 S15L mutation by a second site mutation in ZEN-4 strongly argues that the interaction 10 between CYK-4 and ZEN-4 is critical for CYK-4 function. Indeed, *in vivo*, the majority of ZEN-4 is in a complex with CYK-4. Moreover, three lines of evidence indicate that HsCYK-4 and MKLP1 are in a tetrameric complex. First, immunopurification of HsCYK-4 and MKLP1 reveals 15 equal amounts of the two proteins. Second, these two proteins co-migrate on sucrose density gradients with a similar S value as observed for the *C. elegans* proteins. Third, the two proteins precisely co-migrate on a gel filtration column and their fractionation behavior suggests a native molecular mass for the 20 complex of ~300 kDa. Previous determinations of the native molecular mass of MKLP1 have been reported and the values are similar to those obtained in the present invention here (Chui et al., 2000; Kuriyama et al., 25 1994). Previous studies had not taken the presence of CYK-4 into consideration and therefore they interpreted their data to indicate that MKLP1 exists as a homotetramer. However, the data obtained in the experiments of the invention are not compatible with 30 this interpretation, but they rather indicate that the centalspindlin complex is a tetramer containing two

molecules of the kinesin ZEN-4/MKLP1 and two molecules of the RhoGAP CYK-4.

CYK-4 binds to ZEN-4 in a particularly interesting region of this kinesin family member. Rice et al., 1999 have established that a critical element of the kinesin molecule lies just C-terminal to the catalytic core; the neck linker region. ATP binding to one catalytic core induces a large scale conformational change in the neck linker region that causes the other catalytic core to extend towards the adjacent tubulin dimer situated on the plus side of the initial microtubule contact (Rice et al., 1999). In the present invention, it was found that CYK-4 binds to a region of ZEN-4 that includes the neck linker region. In conventional kinesin, the neck linker region corresponds to a region 15 amino acids long that connects the catalytic core of kinesin to the coiled coil stalk domain. Among the family of KIN-N motors, the MKLP1 subfamily has a distinctly divergent neck linker region, it lacks several nearly invariant residues and the linker between the catalytic core and the coiled coil region is about 5 times longer than in other members on the KIN-N family. The divergence of this critical region of the kinesin suggests that MKLP1-mediated microtubule motility may differ from that of other kinesins. Moreover, since CYK-4 binds to the neck linker region of ZEN-4 it is possible that CYK-4 binding may in fact regulate ZEN-4 motor activity.

Previous work had suggested that MKLP1 may exist as a 30 tetramer containing 4 motor domains (Chui et al., 2000; Kuriyama et al., 1994). If the two dimers were arranged

in an antiparallel orientation, this arrangement could easily explain how this complex could crosslink antiparallel microtubules. However, the data obtained in the present invention are not consistent with this 5 proposed structure. The complex that has been characterized appears to contain two kinesin motors and two RhoGAP molecules. Since most kinesin motors are dimers in which both catalytic cores interact with a single microtubule protofilament, the architecture that 10 is described here can not easily explain how microtubule bundling is achieved. If the two kinesin subunits of centralspindlin bind to the same microtubule protofilament, then how might microtubule crosslinking occur? At this point, at least three 15 possible mechanisms can be envisaged (fig. 18).

The first possibility is that there is an additional microtubule binding site elsewhere in the CYK-4/ZEN-4 complex. No additional binding site has been identified yet in CYK-4 nor has MKLP1, ZEN-4, or Pav 20 been found to have an additional microtubule binding site. However, it has been shown, and was confirmed by the inventors, that MKLP1 interacts differently with microtubules than most kinesin-like proteins. Specifically, ATP is usually sufficient to elute most 25 kinesins from microtubules, but in the case of MKLP1, both ATP and high salt are required (Kuriyama et al., 1994; Nislow et al., 1992). Thus it is possible that MKLP1 interacts with two microtubules, one by the motor domain and another via a different interaction surface. 30 Consistent with this possibility is the finding that Rab6KIFL kinesin, which is quite similar to MKLP1 in primary structure as well as in its localization and

proposed function (Fontijn et al., 2001; Hill et al., 2000), has been reported to contain a second microtubule binding activity in the C-terminal half of the molecule (Echard et al., 1998). However this 5 possibility does not explain why CYK-4 is required for central spindle assembly.

The second alternative is that MKLP1 forms higher order structures and that the tetramer that has been characterized in the present invention is merely a 10 building block. This possibility gains some support from localization studies. In both *C. elegans* and in mammalian cells, ring-like structures are found that are termed division remnants that persist in the cell cortex after division. These remnants appear to be 15 large aggregates of centralspindlin which are not in direct association with microtubules. Higher order oligomers could potentially form in early anaphase and promote microtubule bundling.

The third possibility is that, unlike most N-terminal 20 kinesins, the two catalytic cores of the kinesin subunits in the centralspindlin complex could bind to different microtubules. This would not be without precedent in that the KIN-N KIF1A moves processively along a microtubule using a single head (Okada and 25 Hirokawa, 2000). The association of the two catalytic cores of MKLP1 with different microtubules is made feasible by the fact that the linker region between the catalytic core and the coiled coil domain is much longer than that present in most N-terminal kinesins. 30 Perhaps CYK-4 ensures that the two motor domains are oriented in such a way to bind to antiparallel

microtubules (fig 18). Structural analysis and biochemical reconstitution of centalspindlin from purified components will allow further insight into mechanism of antiparallel microtubule bundling.

5 One critical question, which has not be rigorously explored to date is the function of the central spindle during the process of cytokinesis. The obtained data do not shed extensive new light on this question. However, it may be noted that the RhoGAP domain of

10 CYK-4 is highly conserved at the primary sequence level. Interestingly CYK-4 is significantly less active at inducing GTP hydrolysis by RhoA as compared to Rac and Cdc42 (Jantsch-Plunger et al., 2000), and this feature of CYK-4 is conserved in HsCYK-4 (Toure et

15 al., 1998). It has been proposed by the inventors that one function of the central spindle is to concentrate CYK-4 to a position in the cell where it could promote inactivation of RhoA at a late stage in cytokinesis. Consistent with this model, inactivation of RhoA by

20 RNAi does not prevent CYK-4 localization nor does it impair central spindle assembly. In addition, the RhoGAP domain seems to be required at some stage of cytokinesis, since overexpression of a HsCYK-4/MgcRacGAP bearing a mutation in the RhoGAP domain

25 causes cytokinesis defects (Hirose et al., 2001). Further structure-function analysis of CYK-4 is necessary to test this model further. However such analyses must take into consideration that CYK-4 is able to dimerize or oligomerize and therefore such

30 analysis is best done in the absence of endogenous CYK-4.

Although centalspindlin appears to be a simple tetrameric complex, there are several lines of evidence that suggest that it may be subject to regulation by at least two different kinases. Genetic analysis of Polo 5 kinase in *Drosophila* indicates Pav localization is Polo dependent (Carmena et al., 1998) and moreover a biochemical interaction between Polo and MKLP1 orthologs have been shown in extracts from both mammalian cell and *Drosophila* embryos (Adams et al., 10 1998; Lee et al., 1995). In addition, the Aurora-B/Incenp complex is also required for the stable localization of centalspindlin in *C. elegans* embryos (Kaitna et al., 2000; Schumacher et al., 1998; Severson et al., 2000) and an in vitro biochemical interaction 15 has been detected between Aurora-B (AIR-2) and ZEN-4 (Severson et al., 2000).

A stable association between Polo kinase or Aurora-B kinase with centalspindlin has not been observed. Reconstitution of central spindle assembly in vitro 20 will allow the role of these and other regulators of centalspindlin to be dissected.

In summary, the experiments of the present invention comprise an initial phenotypic, molecular, biochemical, and cell biological analysis of the *cyk-4* gene. 25 Furthermore, they comprise an analysis of the CYK-4/ZEN-4 complex (centalspindlin) that consists of two molecules of each CYK-4 and ZEN-4.

The experiments of the present invention (Examples 1-5) indicate that Cyk-4 is an active GTPase activating 30 protein that is required for cytokinesis, likely by its

ability to regulate the RhoA GTPase. Quite surprisingly, one additional function of this protein is to promote assembly of the central spindle. Cyk-4 is a key molecule required for cytokinesis that regulates 5 both the structure of the late mitotic spindle and the function of the contractile ring.

Thus, in a first aspect, the present invention generally relates to Cyk-4 polypeptides, which have been shown to be essential for this second microtubule 10 dependent step and thus define a functional link between the central spindle and the contractile ring.

In a preferred embodiment, the invention relates to a mammalian Cyk-4 polypeptide, in particular murine and human Cyk-4.

15 In a first aspect, the invention relates to the murine Cyk-4 polypeptide with the amino acid sequence as set forth in SEQ ID NO:4 or with the amino acid sequence encoded by a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a 20 nucleotide sequence as set forth in SEQ ID NO:3.

In a preferred aspect, the invention relates to the human Cyk-4 polypeptide with the amino acid sequence as set forth in SEQ ID NO:2 or with the amino acid sequence encoded by a polynucleotide which hybridizes 25 under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:1.

In a further aspect, the present invention relates to an isolated DNA molecule comprising a polynucleotide with the nucleotide sequence as set forth in SEQ ID

NO:1 encoding human Cyk-4 polypeptide, or an isolated DNA molecule encoding human Cyk-4 polypeptide comprising a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a 5 nucleotide sequence as set forth in SEQ ID NO:1.

In a further aspect, the present invention relates to an isolated DNA molecule comprising a polynucleotide with the nucleotide sequence as set forth in SEQ ID NO:3 encoding murine Cyk-4 polypeptide, or an isolated 10 DNA molecule encoding murine Cyk-4 polypeptide comprising a polynucleotide which hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO:3.

By "stringent hybridization conditions" as used herein 15 is meant overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (1X SSC = 150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, 20 followed by washing the filters in 0.1x SSC at about 65°C, or equivalent conditions.

In the following, if not otherwise stated, the term "Cyk-4" refers to both the murine and the human Cyk-4.

Homologues of the subject Cyk-4 polypeptides also 25 include versions of the polypeptide which are resistant to post-translation modification or which alter the activity of the protein. Cyk-4 polypeptides may comprise a full length protein, such as represented in SEQ ID NO:2 or 4, or they may comprise fragments or variants 30 therof.

Beside DNA molecules, the present invention relates to nucleic acid molecules in the form of RNA, such as mRNA. The DNA molecules include cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA

5 may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense (or plus) strand, or it may be the non-coding strand, also referred to as the antisense (or minus) strand. The present invention also

10 relates to preparations of double stranded Cyk-4 RNA or derivatives thereof that can be used to interfere with gene expression by ds-RNA mediated gene interference as described by Fire et al. 1998 and reviewed by Fire, 1999; Bosher and Labouesse, 2000; Sharp, 1999.

15 By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. Recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further

20 examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells, and those DNA molecules purified (partially or substantially) from a solution whether produced by recombinant DNA or synthetic chemistry techniques.

25 Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. However, it is intended that "isolated" as used herein does not include the Cyk-4 cDNA present in a cDNA library or in a preparation of purified or

30 isolated genomic DNA containing the *Cyk-4* gene or a portion thereof in admixture with one or more other cDNA molecules or DNA fragments.

The nucleic acid molecules of the present invention further include genetic constructs comprising one or more Cyk-4 DNA sequences operably linked to regulatory DNA sequences (which may be heterologous regulatory sequences), such as promoters or enhancers, wherein upon expression of these DNA sequences in host cells, preferably in bacterial, fungal (including yeast), plant or animal (including insect or mammalian) cells, one or more *Cyk-4* polypeptides are produced. In such constructs, the design of which is described in laboratory manuals (see e.g. Sambrook et al., 2000) and is routine to the skilled artisan, the regulatory sequences may be operably linked to a Cyk-4 polynucleotide encoding mature *Cyk-4* polypeptide or any of its variants, precursors, fragments or derivatives described herein, which may include one or more polynucleotides having a nucleic acid sequence that is complementary to substantially all or a portion of a nucleic acid molecule having a nucleic acid sequence as shown in SEQ ID NO:1 or 3. As used herein, the terms "a portion" or "a fragment" of a nucleic acid molecule or a polypeptide means a segment of a polynucleotide or a polypeptide comprising at least 15, and more preferably at least 20, contiguous nucleotides or amino acids of a reference polynucleotide or polypeptide (for example, the polynucleotide and polypeptide shown in SEQ ID NOs: 1 or 3, respectively, unless otherwise specifically defined below.)

Besides the DNA molecules having a nucleotide sequence corresponding to that depicted SEQ ID NO:1 or 3, the invention also relates to DNA molecules which comprise a sequence substantially different from those described

above but which, due to the degeneracy of the genetic code, still encode the Cyk-4 mouse or human polypeptides. Since the genetic code is well known in the art, it is routine for one of ordinary skill in the 5 art to produce the degenerate variants described above without undue experimentation.

In addition, the invention relates to Cyk-4 polypeptides which have deviations from the sequence shown in SEQ ID NO:2 or 4, caused by the conservative 10 exchange of amino acids, if they are Cyk-4 derivatives or fragments or peptides with the properties which are desirable for their use in therapy or in screening assays, or isolated DNA molecules encoding such derivatitves or fragments with a polynucleotide 15 sequence varying in their sequence from SEQ ID NO:1 or 3.

Nucleic acid molecules of the present invention which encode a Cyk-4 polypeptide or a derivative or fragment thereof may include, but are not limited to, those 20 encoding the amino acid sequence of the polypeptide by itself, together with additional, non-coding sequences, including for example introns and non-coding 5' and 3' sequences, such as the transcribed, untranslated regions (UTRs) or other 5' flanking sequences that may 25 play a role in transcription (e.g., via providing ribosome- or transcription factor-binding sites), mRNA processing (e.g. splicing and polyadenylation signals) and stability of mRNA; the coding sequence for the Cyk-4 polypeptide operably linked to a regulatory DNA 30 sequence, particularly a heterologous regulatory DNA sequence such as a promoter or enhancer; and the coding

sequence for the *Cyk-4* polypeptide linked to one or more coding sequences which code for amino acids that provide additional functionalities. Thus, the sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a peptide which facilitates purification of the fused polypeptide. In certain embodiments of this aspect of the invention, the marker amino acid sequence may be a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described for instance in Gentz et al., 1989, hexa-histidine provides for convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson et al., 1984. Yet another useful marker peptide for facilitation of purification of *Cyk-4* is glutathione S-transferase (GST) encoded by the pGEX fusion vector (see, e.g., Winnacker, From Genes to Clones, New York: VCH Publishers, pp. 451-481 (1987)). As discussed below, other such fusion proteins include the *Cyk-4* fused to immunoglobulin Fc at the N- or C-terminus.

A still further aspect of the present invention relates to antibodies and antibody preparations specifically reactive with an epitope of the *Cyk-4* polypeptide. Polyclonal antibodies are conventionally obtained by immunising animals, particularly rabbits, by injecting the antigen or fragments thereof and subsequently purifying the immunoglobulin.

Monoclonal anti-Cyk-4-antibodies may be obtained by standard procedures following the principle described by Köhler and Milstein, 1975, by immunising animals, particularly mice, then immortalising antibody-  
5 producing cells from the immunised animals, e.g. by fusion with myeloma cells, and screening the supernatant of the hybridomas obtained by immunological standard assays for monoclonal anti- Cyk-4 -antibodies. For therapeutic or diagnostic use in humans, these  
10 animal antibodies may optionally be chimerised in the conventional way (Neuberger et al., 1984, Boulianne et al., 1984) or humanised (Riechmann et al., 1988, Graziano et al., 1995).

Cyk-4 specific antibodies can be used for both  
15 diagnostic and screening applications.

Cyk-4 specific antibodies are useful in clinical situations, where determining overexpression or underexpression of CYK-4 has predictive value. Anti-CYK-4 antibodies can be used on tissue or cell specimens to evaluate the level of expression or changes in the subcellular localization in various disease states. In addition, anti-CYK-4 antibodies can be used to detect the presence of CYK-4 in solid-phase binding assays as described in the subsequent  
20 subsection related to finding compounds that affect the interaction of CYK-4 with other proteins.  
25

In a further aspect, the present invention provides screening methods for identifying compounds capable of modulating, in particular inhibiting, cytokinesis by

modulating, in particular inhibiting, the function of CYK-4.

In a first embodiment, the screening method of the invention is used to identify compounds that modulate 5 the function CYK-4 to promote GTP hydrolysis by Rho family GTPases. (Due to the evolutionary conservation of the GTPase domain and the requirement for Rho in cytokinesis, it can be concluded from the findings of the invention that the ability of CYK-4 to promote GTP 10 hydrolysis by Rho is crucial for CYK-4 function.)

In this embodiment of the invention, a compound's ability to promote GTP hydrolysis by a Rho family GTPase is determined by incubating a substrate selected from the members of the Rho family GTPases with GTP for 15 a period of time sufficient to allow saturation of the substrate's GTP binding sites, adding Cyk-4 and allowing it to react in the presence or absence of the test compound, and determining the amount of hydrolyzed GTP.

20 This type of assay may be conducted on the basis of the so-called "GTP hydrolysis assay", which is a biochemical assay carried out according to standard protocols, as described, *inter alia*, by Self et al., 1995, or by Settleman and Foster, 1995. By way of 25 example, this assay may be carried out as follows: In a first step, the substrate is incubated with GTP that carries a radioactive label (e.g. like the commercially available  $\gamma$ -<sup>32</sup>P-GTP, or an otherwise measurable, e.g. a fluorescent label, as described, *inter alia*, by Hazlett 30 et al., 1993, under conditions and for a period of time

sufficient to allow saturation of GTP binding sites. Subsequently, CYK-4 is added, optionally at various concentrations of from 0 to e.g. 1 uM; in the presence of absence of test compounds. After the appropriate time 5 (e.g. 5 min.) the amount of GTP that has been hydrolysed is determined.

After the time required for CYK-4 stimulated GTP hydrolysis by Rho in the control reaction (absence of test compound), the reaction solution may be filtered 10 through a protein binding matrix, e.g. nitrocellulose, that is arranged in the same geometrical pattern as the original microtiter plate where the reaction took place, and the radioactivity retained in the filters, or the amount of liberated free phosphate (or other 15 label as appropriate) is quantified.

To simplify the screening assay, the Rho protein may be immobilized on a solid matrix, either via a tag that allows for binding to a suitably modified solid support, e.g. by using a biotinylated Rho protein and a 20 streptavidin-coated microtiter plate, the solid matrix may also be in the form of beads. In the above-described assays that measure GTP hydrolysis, the Rho protein is preferably human.

By way of example, the assay may be conducted as 25 follows: A member of the Rho family GTPases is immobilized on a solid support, e.g. a SPA (proximity scintillation assay bead) using either an epitope tag such as His6 or other similar tags. The Rho protein is loaded with radioactive  $^{32}\text{P}$ -gamma-GTP in the presence of 30 EDTA. Magnesium and Cyk-4 are added in the absence or

presence of test compounds, incubated for various times at which point the amount of radioactivity or other suitable label remaining associated with the beads is measured. Other embodiments of the assay include using 5 alternative labels such as fluorescent GTP or other radioisotopes and using alternative measures of GTP hydrolysis such as measuring the amount of free  $^{32}\text{P}$  released, as described above.

Alternatively to using the full-length Rho protein, a 10 truncated version may be used, as long the GTPase activity is maintained, e.g. a fragment that lacks the critical cysteine residue in the CaaX box at the C-terminus that is required for membrane targetting.

A number of Rho proteins are available that are 15 suitable for use in a GTP hydrolysis assay as a substrate, they have been cloned from various species. Preferably, they are employed as recombinant proteins.

Examples for Rho substrates that may be employed are human RhoA (Swiss Prot Primary Accession Number 20 P06749), human RhoB (Swiss Prot Primary Accession Number P01121), human RhoC (Swiss Prot Primary Accession Number P08134), human RAC1 (Swiss Prot Primary Accession Number P15154), human RAC2 (Swiss Prot Primary Accession Number P15153), human RAC3 25 (Swiss Prot Primary Accession Number O14658) and human GB25 (Swiss Prot Primary Accession Number P21181).

Based on the sequence information, the Rho substrates and the potentially present co-factor(s) can be produced recombinantly in suitable host cells, e.g. in

E. coli or by means of baculovirus in insect cells, according to standard methods.

Compounds that have the ability to prevent CYK-4 stimulated GTP hydrolysis by Rho family GTPases are 5 then tested for specificity in a similar assay format using other proteins that have Rho family GAP domains, e.g. p190 or RhoGAP (Lamarche and Hall, 1994).

In an alternative embodiment of the screening method, the compound's ability to inhibit Cyk-4 function is 10 determined by determining the compound's ability to interfere with the biochemical interaction of CYK-4 with a member of the MKLP1 subfamily.

The function of CYK-4 in cytokinesis is intimately tied to its ability to interact with MKLP1, a kinesin like 15 protein that is also required for central spindle assembly. Therefore compounds that inhibit this interaction are expected to prevent cytokinesis and to inhibit cell proliferation.

Examples for members of the MLKP1 family are CeM03D4.1b 20 (C. elegans; GenBank ID U61955, Protein ID 1397342) and HsMKLP1 (human; GenBank ID X67155; SwissProt Q02241). Preferably, HsMKLP1 is employed.

In C.elegans extracts, CYK-4 and CeMKLP1 are present in a protein complex and can be co-immunoprecipitated and 25 co-purified. The nature of this protein interaction can be further characterized, in particular by determining whether additional proteins also interact with this complex.

In an assay that employs an additional protein component involved in this interaction, a compound's ability to modulate Cyk-4 function is determined by determining its effect on the interaction of said 5 protein with the Cyk-4/MKLP1 complex.

In carrying out an assay based on the Cyk-4/MKLP1 interaction, a minimal set of proteins necessary to form a stable complex, i.e. Cyk-4, MKLP1, and 10 additional potential protein co-factors are produced in recombinant form.

The domains required for the interaction of Cyk-4 and MKLP1 have been identified by the present inventors.

In an embodiment of the invention, the domain of Cyk-4 that interacts with MKLP1 (i.e. the N terminal Cyk-4 15 region containing at least amino acids 1-120), or the whole Cyk-4 protein, is immobilized on a solid support, as described below. The domain of MKLP1 that interacts with CYK-4 (i.e. the central MKLP1 domain containing at least amino acids 503-603) or the whole MLKP1 protein 20 that is modified with a suitable label to allow for rapid detection (i.e. radiolabelled, fluorescently labeled, hapten labelled etc., as described below) is incubated in the presence or absence of the test compounds.

25 After an incubation period that allows for interaction of the proteins, e.g. for about 20 minutes at 25°C, the amount of MKLP1 bound to the immobilized CYK-4 is measured by use of the label outlined above or by the use of suitable antibodies in an ELISA type assay. The

assay may also be setup in the reverse, e.g. with Cyk-4 being labeled or with MKLP1 immobilized, or by performing the binding reaction in solution and then capturing one of the components on a solid support and

5 measuring the amount of the other component that is co-immobilized.

According to another aspect, the invention relates to a screening method that takes advantage of the biochemical multimerization of CYK-4 on the one hand

10 and the biochemical multimerization of the MKLP1 subfamily of the kinesin-like protein superfamily on the other hand. This approach is based on the finding that the domain of CYK-4 that is required for interaction with MKLP1 contains a coiled-coil domain

15 that mediates self association. Likewise the domain of MKLP1 that is required to interact with CYK-4 contains a coiled-coil domain that mediates self association.

Since the ability of these proteins to self association is expected to be required for their function *in vivo*,

20 compounds that inhibit the self association of either one or both of the interaction partners Cyk-4 and MKLP1 are candidates for perturbing cytokinesis.

In an embodiment of the invention that is based on determining whether a test compound has the ability to

25 interfere with the self association of Cyk-4, the N-terminal domain of CYK-4 (containing at least amino acids 1-120), or the whole Cyk-4 protein, is immobilized on a solid support, either directly or through a tag. (Suitable tags are commercially

30 available, e.g. the FLAG, HA, MYC, HIS tag, etc.).

Examples for solid supports useful in the present invention are commercially available immunobeads or immunoplates, e.g. 96-well immunoplates, or microchips, which are coated with an antibody directed against one 5 of the above-listed tags fused to the interacting protein.

A second CYK-4 fragment consisting of or containing the N terminal domain of CYK-4, i.e. amino acids 1-120, or the whole Cyk-4 protein is modified with a suitable 10 label that allows for rapid detection. (The immobilized and the labeled Cyk-4 protein (fragments) may be identical or different.)

Examples for suitable labels are commercially available radioactive or fluorescence labels, hapten labels, or 15 peptide labels, e.g. Europium or the Green Fluorescent Protein (GFP), an enzyme label, e.g. luciferase, alkaline phosphatase etc.)

The labeled Cyk-4 (fragment) is incubated with the immobilized CYK-4 in the presence or absence of the 20 test compounds.

After an incubation period that allows for interaction of the proteins, e.g. for ca. 20 min, the amount of CYK-4 bound to the immobilized CYK-4 is determined by measuring the signal of the label or by the use of 25 suitable antibodies in an ELISA type assay.

In a further embodiment of the invention, the screening method to identify modulators of Cyk-4 function is based on determining whether a test compound has the ability to interfere with the self association of

A P P E N D I X

MKLP1. In this embodiment, the central domain of MKLP1 (containing at least amino acids 503-603) or the whole MKLP1 protein, is immobilized on a solid support.

A second MKLP1 fragment, consisting of or containing 5 the above-defined central domain of MKLP1, or the whole MKLP1 protein is modified with a suitable label that allows for rapid detection. (The immobilized and the labeled MKLP1 protein (fragments) may be identical or different).

10 The assay for identifying inhibitors of MKLP1 self assembly may be conducted in the the same way as described above for identifying inhibitors of Cyk-4 self assembly.

15 All embodiments of the screening methods of the invention may be performed in the high throughput format by automation of the reaction steps. In this case, a great number of compounds, e.g. from compound or natural product libraries, are applied to microtiter plates containing the components necessary for the 20 reaction.

Due to their ability of inhibiting the function of Cyk-4 to promote GTP hydrolysis by Rho family GTPase, of interfering with the interaction between Cyk-4 and MKLP1, or by interfering with self assembly of Cyk-4 25 and/or MKLP1, respectively, compounds identified in the above screens have the potential to perturb cytokinesis. In tumor cells, this effect may result in a decrease or a stop of tumor growth. In addition, inhibition of cytokinesis may cause the activation of a

cell cycle arrest check point that will trigger apoptosis of the tumor cells.

In light of the recent observation (Van de Putte et al 2001) that murine CYK-4 is highly expressed in Purkinje 5 cells of the brain, in particular in cells that have abundant antiparallel bundles of microtubules (as is the case in the central spindle), it may be assumed that compounds that inhibit CYK-4 function in cytokinesis may also impair CYK-4 function in neuronal 10 cells. Inhibitors of CYK-4 function in these cells may have utility in the treatment of neurological disorders, e.g. neuronal hyperactivity.

Compounds that have been identified in the above-described assays to specifically affect CYK-4 function 15 are drug candidates which may be further developed, e.g. in a first step, by structure-function analysis.

To further evaluate the potential of the compounds as drugs, the candidate compounds can be assayed for their effect on cytokinesis and other cellular processes in 20 tissue culture of normal or transformed cells. To test the inhibition of tumor cell proliferation, primary human tumor cells, are incubated with the compound identified in the screen and the inhibition of tumor cell proliferation is tested by conventional methods, 25 e.g. bromo-desoxy-uridine or  $^3\text{H}$  thymidine incorporation.

Compounds that exhibit an anti-proliferative effect in these assays may be further tested in tumor animal models and used for the therapy of tumors.

Thus, in a further aspect, the invention relates to compounds identified in the above screens for the therapy of tumors and any other situation in which cell overproliferation is observed.

5    Toxicity and therapeutic efficacy of the compounds identified as drug candidates by the screening methods of the invention can be determined by standard pharmaceutical procedures, which include conducting cell culture and animal experiments to determine the

10    IC50, LD50, the ED50. The data obtained are used for determining the human dose range, which will also depend on the dosage form (tablets, capsules, aerosol sprays, ampules, etc.) and the administration route (oral, buccal, nasal, parenteral or rectal). A

15    pharmaceutical composition containing the compound as the active ingredient can be formulated in conventional manner using one or more physiologically active carriers and excipients. Methods for making such formulations can be found in manuals, e.g. Remington Pharmaceutical Sciences, 1980; Remington: The Science and Practice of Pharmacy, 2000.

Brief description of the figures.

Figure 1:

5 *cyk-4(t1689ts)* mutant embryos fail to complete cytokinesis.

Figure 2:

*cyk-4* mutant embryos produce deeply ingressing cleavage furrows, but do not form a prominent central spindle.

10 Figure 3:

Positional cloning of the *cyk-4* locus.

Figure 4:

*cyk-4(RNAi)* causes disorganization of the proximal gonad and affects formation of the central spindle.

15 Figure 5:

CYK-4 enhances GTP hydrolysis by Rho, Rac, and Cdc42.

Figure 6:

*rho(RNAi)* causes cytokinesis defects and *cdc42(RNAi)* causes defects in spindle positioning.

20 Figure 7:

CYK-4 localizes to the central spindle and division remnants.

Figure 8:

Time lapse analysis of CYK-4:GFP.

Figure 9:

5 CYK-4 and ZEN-4/CeMKLP1 colocalize and are interdependent for their localization.

Figure 10:

Model for the function of CYK-4 in central spindle formation and cytokinesis.

Figure 11:

RhoA does not have a role in the formation of central spindle

Figure 12:

5 CYK-4 and ZEN-4 associate in vivo and in vitro

Figure 13:

The N- terminus of CYK-4 is necessary and sufficient to bind to ZEN-4

Figure 14:

10 The central region of ZEN-4 is necessary and sufficient to bind to CYK-4

Figure 15:

A complex of CYK-4 and ZEN-4 is required for function in vivo

15

Fiure 16:

Self-association of CYK-4 and ZEN-4

Figure 17:

A complex of MKLP-1 and HsCYK-4 in HeLa cells

5 Figure 18:

Centralspindlin and its role in the formation of  
central spindle

In the Examples 1 - 5, if not otherwise stated, the

10 following materials and methods were used:

a) Strains and alleles

The *cyk-4(t1689ts)* allele was identified in a search  
for maternal effect lethal mutations on chromosome III  
15 (see (Gönczy et al., 1999b) for details). The strains  
DR104, BW1535, BW1369, and RW7000 were obtained from  
the CGC (C.elegans Genetics Center). The strain EU699  
containing the *zen-4(or153ts)* allele was described by  
Severson, et al., 2000. The end points of the  
20 deficiency tDf10 (Heinke Schnabel, unpublished data)  
are not molecularly defined, but it uncovers *cyk-4*,  
*lit-1*, and *bli-5* and it does not uncover *unc-64*.

## b) Antisera

Cyk-4 specific antisera were produced in rabbits using a His6-Cyk-4 fusion protein as immunogen (containing amino acids 407-613 of cyk-4). A GST-Cyk-4 fusion containing amino acids 407-681 of Cyk-4 was coupled to a Hi-Trap NHS resin (Amersham-Pharmacia) and used to affinity purify anti-Cyk-4 antibodies which were used at a final concentration of 1:300. The antibodies used for the studies are specific for Cyk-4 since the staining can be blocked with antigen, a similar pattern is observed when anti-GFP antibodies are used to detect a Cyk-4:GFP fusion construct, and the staining pattern is disrupted in cyk-4 mutant embryos. The rat monoclonal YOL 1/34 anti-tubulin antibody was used at a dilution of 1:200-500. Anti-GFP antibodies (Roche) were used at a dilution of 1:500. Antisera specific for Zen-4/MKlp1 was generously provided by Bill Saxton and Susan Strome (Univ. of Indiana) and used at a dilution of 1:4000. Antisera specific for Air-2 was generously provided by Andy Golden (NIH) and used at a dilution of 1:1000.

## c) Genetic mapping of Cyk-4

The *cyk-4* locus maps under the deficiency tDf6 which deletes a large fraction of the distal right arm of LGIII. Recombination mapping using *unc-32(e189)* *cyk-4(t1689ts)/dpy-18(e364)* *unc-25(e156)* placed *cyk-4* distal to (or very close to) *unc-25* (23/23 Dpy non Unc's carried the *cyk-4* mutation). Recombination between *dpy-18 cyk-4/RW7000* which carries several Tc1

elements including one on the cosmid F14F7 gave rise to 45 Dpy non-Ts animals, 3 of which lacked the TC1 insertion on F14F7, indicating that the *cyk-4* gene is distal to this cosmid. Crosses to strains carrying the 5 deficiencies ctDf3, ctDf2, tDf10 revealed that *cyk-4(t1689ts)* is not uncovered by ctDf3 and is uncovered by both ctDf2 and tDf10. Since tDf10 does not uncover *unc-64*, *cyk-4(t1689ts)* must be distal to *unc-64*.

10

d) Time Lapse recordings

Time lapse Nomarski imaging was performed as described previously (Jantsch-Plunger and Glotzer, 1999). Time lapse imaging of Cyk-4:GFP was performed on a Zeiss 15 Axiovert microscope using a 100X/1.3 neofluar objective. The illumination source, an Atto-arc HBO-103, was reduced to 50% intensity. An intensified cooled CCD camera (GenIV pentamax, Princeton Instruments) was used for image acquisition. The camera 20 and other electronics were controlled with MetaMorph software (Universal Imaging). Typical acquisition times were 40-80 msec. Each 10 seconds, 4-5 fluorescent images were acquired at different focal planes and a nomarski image was acquired. The fluorescent images 25 were projected onto a single frame using the maximum intensity from the stack of images. Under these conditions, embryos could be filmed for more than one hour without affecting the cell cycle timing or pattern of cell divisions.

30

## e) Rescue Experiments

To identify the *cyk-4* gene in this region cosmid DNA (from stocks kindly provided by Alan Coulson, Sanger Center) was coinjected with the *rol-6(su1006)* dominant marker (Mello et al., 1991) into the gonad of *unc-32 cyk-4/qC1* worms. Heterozygous F1 hermaphrodites that carried the *rol-6* dominant marker were cloned to individual plates at 25°C and the presence of Unc progeny, indicating zygotic rescue of the *cyk-4* mutation, was assessed. Individual *unc-32, cyk-4* worms carrying the extrachromosomal arrays were cloned to individual plates to assess the extent of germline rescue. The *cyk-4* genomic rescue construct MP17, contains a 4.9 kb genomic *Xba*I fragment excised from K08E3 and inserted into pBS-KS+.

## f) RNA interference

Approximately 500 bp of DNA corresponding to predicted coding regions of Rho (Y51H4A.B), Rac-1 (C09G12.8B), Cdc42 (R07G3.1), F22E12.2, Y32F6B.3, K08D3.9, K08E3.2, K08E3.3, K08E3.4, K08E3.6, K08E3.7, K08E3.8 were amplified by PCR and cloned into pGEM-T (Promega). Double stranded RNA was transcribed (Ambion) and annealed, and injected into the gonads of wildtype N2 hermaphrodites as described (Fire et al., 1998).

## g) Production of Cyk-4:GFP transgenes

The GFP cassette from vector pPD119.16 was excised with BspLUIII and inserted into the unique NcoI site of MP17 (see above). This construct was linearized with XbaI, 5 and complex arrays containing linearized genomic DNA and linearized *rol-6*(su1006) DNA were mixed in a ratio of 1:100:1 and injected into *unc-32*(e189) *cyk-4*(*t1689ts*)/*qC1* hermaphrodites. Rolling F1 heterozygotes were singled out at 25°C and rolling Unc 10 F2 animals were picked. A line MG110, was obtained that gave stable rescue of the *cyk-4*(*t1689ts*) mutation.

The structure of the *cyk-4* gene was established by analysis of a large number of EST sequences available in the sequence databases at the Sanger Center and the 15 National Institute of Genetics and by sequencing the clones yk63D6 and yk104g12 (kindly provided by Yuji Kohara). The structure of the gene is identical to the structure predicted by the *C. elegans* genome consortium.

20

## h) Immunolocalization

Immunolocalization studies were performed as previously described (Jantsch-Plunger and Glotzer, 1999). Briefly, gravid hermaphrodites were placed on 25 aminoproyl-silane treated slides, a coverslip was added, and sufficient pressure to extrude the embryos was applied. The slide was placed into liquid nitrogen. The coverslip was removed while the sample was still frozen, the preparation fixed with -20°C

methanol and then antibody staining was performed according to standard procedures.

i) Biochemical analysis of Cyk-4

5 The coding regions of Rho, Rac, and Cdc42 were PCR amplified and cloned into pET28b with an C-terminal polyhistidine tag. The GTPases were expressed at 25°C and purified using Ni<sup>2+</sup>-NTA-agarose (QIAGEN). Proteins were dialyzed into 50 mM Tris pH 7.5, 50 mM NaCl, 5 mM  
10 MgCl<sub>2</sub> and quick frozen. The GAP domain of Cyk-4 (amino acids 407-681) was cloned into pGEX4T-1. Proteins were expressed at 25°C and purified using GSH-agarose (Sigma). Proteins were dialyzed into 50 mM Tris pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT and quick frozen.  
15 To assess GAP activity, 15 pmol of the GTPases were loaded with 1 pmol <sup>32</sup>P- $\alpha$ -GTP in 20 mM Tris pH 7.6, 4 mM EDTA, 25 mM NaCl, 1 mM DTT, 1 mM ATP, 0.1 mg/ml BSA at room temperature. The sample was place on ice and MgCl<sub>2</sub> was added to 17 mM. GST-Cyk-4-GAP was added at the  
20 indicated concentrations and, at intervals, samples were taken by dilution into 2% SDS, 20 mM EDTA. Aliquots were spotted onto TLC plates (PEI-cellulose, Machery-Nagel) and developed in 1M LiCl. The plates were dried and exposed using a Storm Phosphoimager  
25 (Molecular Dynamics) and the data analysis was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health; <http://rsb.info.nih.gov/nih-image/>).

## Example 1

cyk-4 mutants initiate, but do not complete, cytokinesis

The *cyk-4(t1689ts)* allele was isolated in a screen for 5 maternal effect embryonic lethal mutations on chromosome III (Gönczy et al., 1999b). The *cyk-4* locus is defined by a single, temperature sensitive (ts), allele. The phenotype of embryos derived from homozygous *cyk-4* hermaphrodites at the restrictive 10 temperature (hereafter referred to as *cyk-4* mutant embryos) during the first division is shown in figure 1B. *cyk-4* mutant embryos appear normal until cytokinesis, except that defects in polar body extrusion are frequently observed (data not shown). 15 The first cleavage furrow forms at the correct time and place, it ingresses extensively, but, invariably, it regresses. Quantitation of the extent of furrow ingress in *cyk-4* mutant embryos reveals that, on average, furrows ingress to 73+/-13% (n=14) of the egg 20 diameter. A multipolar spindle develops in the second cell cycle and the process of furrow ingress and regression occurs again. This pattern is repeated until the embryos become grossly disorganized. (Figure 1 shows that *cyk-4(t1689ts)* mutant embryos fail 25 to complete cytokinesis. Wildtype embryos (A) and embryos from *cyk-4* mutant hermaphrodites (B) were dissected from young adults, mounted on agarose pads and observed by time-lapse nomarski microscopy. The ingressing cleavage furrow is indicated (arrows). 10  $\mu$ m 30 scale bars.)

Although the majority of cell divisions in worm development occur early in embryogenesis, cells of the germline and many cells in the nervous system are produced during post embryonic development (Sulston and Horvitz, 1977). A number of mutations in genes required for cell division cause worms to become sterile and uncoordinated (O'Connell et al., 1998; Woollard and Hodgkin, 1999). To determine if *cyk-4* is required post embryonically, temperature shift experiments with worms homozygous for the *cyk-4(t1689ts)* allele were performed (table 1). Homozygous animals grown at 16°C are viable and fertile. Homozygous *cyk-4* animals shifted to 25°C at the L4 stage produce embryos with the cytokinesis defects described above. Homozygous animals shifted to 25°C at earlier stages either fail to hatch or become sterile and uncoordinated, depending on the time of the temperature shift. Thus *cyk-4* is required postembryonically, perhaps due to its role in cell division. Unexpectedly, animals shifted at the L2/L3 stages become highly uncoordinated adults, even though most motor neurons would be expected to have completed all their divisions at the time of the shift. This may suggest that CYK-4 has additional roles besides its role in cell division. However, not out the possibility cannot be ruled out that some of the cells in the ventral nerve cord divide later in *cyk-4* mutants.

To investigate why *cyk-4* mutant embryos fail to complete cytokinesis, actin and tubulin were localized in wild-type and *cyk-4* mutant embryos. Both wild-type and mutant embryos contained deeply ingressing cleavage furrows that stain with an anti-actin antibody; the

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results are shown in figure 2. Figure 2A,B shows that *cyk-4* mutant embryos produce deeply ingressing cleavage furrows, but do not form a prominent central spindle. Wild type (A, C) and *cyk-4* mutant embryos (B,D) were 5 fixed and stained for actin (green) and DNA (blue) (A,B); tubulin (green) and DNA (blue) (C,D). 10  $\mu$ m scale bars.

The metaphase spindles of *cyk-4* mutant embryos appeared normal. However, spindle morphology during early 10 anaphase was significantly different in mutant embryos as compared to wild type. In wild-type embryos (20/20) prominent microtubule bundles form between the separating masses of chromatin, forming the central spindle (figure 2C). In *cyk-4* mutant embryos (9/10) 15 such bundles were largely reduced and disorganized (figure 2D). It is concluded that CYK-4 is required for the organization of the central spindle during anaphase. Since the central spindle is required for cytokinesis, it is possible that the *cyk-4* mutant 20 embryos fail to complete cytokinesis because they fail to assemble the central spindle.

#### Example 2

##### Cloning of the *cyk-4* gene

25 To investigate the molecular basis for the phenotypes described above, it was first sought to map the *cyk-4* locus and clone the affected gene (see methods for details). The *cyk-4* gene maps distal to *unc-64* on the extreme right arm of LG III. Figure 3 shows the

positional cloning of the *cyk-4* locus. (A) A schematic of LGIII showing the positions of various loci and the extent of the deficiencies. *cyk-4* is uncovered by tDf10, tDf6, and ctDf2, but not ctDf3. (B) An 5 enlargement of the physical map from the *unc-64* locus until the end of LGIII. The ability of various cosmid pools to rescue *cyk-4* is indicated. (C) A map of the predicted genes on K08E3. All the predicted genes except for K08E3.1 and K08E3.5 were inactivated by RNAi 10 and only RNAi of K08E3.6 produced multinucleate embryos. (D) A schematic representation of the domain structure of CYK-4 and its human ortholog. The position of the point mutation identified in *cyk-4* (*t1689ts*) is indicated.

15 The *cyk-4* gene was then identified by functional rescue of the zygotic requirement for *cyk-4* using pools of cosmids. A pool of three cosmids (ZK520, W06F12, and K08E3) allowed *cyk-4* homozygotes to hatch and develop to adulthood at 25°C. These cosmids were injected 20 individually and cosmid K08E3 contained rescuing activity. Further subcloning revealed that a 4.9 kb genomic fragment, predicted to contain the complete K08E3.6 gene and no other intact gene, could rescue the *cyk-4* zygotic and germline phenotypes. Finally, the 25 coding region was amplified from DNA derived from *cyk-4* (*t1689*) homozygotes and sequenced and a single point mutation was identified that differed from the sequence provided by the genome project; this mutation was not observed in another line derived from the same 30 parental strain. These data show that the defect in *cyk-4* (*t1689ts*) embryos is due to a point mutation in the K08E3.6 gene.

The predicted protein product of the *cyk-4* gene has a C-terminal domain that contains the consensus motifs of GTPase activating proteins for Rho family GTPases (figure 3). Adjacent to the C-terminal GAP domain is a 5 C1 domain that is predicted to bind to diacylglycerol or phorbol esters. At the amino terminus of the protein is a 90 amino acid region predicted to form a coiled-coil domain. The S15L point mutation found in *cyk-4(t1689ts)* , is located just amino terminal to the 10 coiled-coil domain. Human and mouse proteins with structural similarities to *cyk-4* have been described (Toure et al., 1998; Wooltorton et al., 1999). These genes are expressed in a variety of proliferating tissues. In addition, the *Drosophila* sequence database 15 contains an entry (ascension no. AC005977 (CLOT 94)) that, together with CYK-4 and the previously mentioned human gene, share a common structure consisting of approximately 650 amino acids, an NH<sub>2</sub>-terminal coiled-coil domain and a conserved C-terminus containing C1 20 and GAP domains. The structural conservation of CYK-4 suggests that its function is conserved among metazoans.

In the present invention, the the human and mouse cDNA's were identified by searching the DNA databases 25 with the *C.elegans* CYK-4 protein sequence.

Since the *cyk-4(t1689ts)* mutation may contain residual activity RNA-mediated interference (RNAi) was used to deplete embryos of CYK-4 protein.

Figure 4 shows that *cyk-4(RNAi)* causes disorganization 30 of the proximal gonad and affects formation of the

central spindle. Young adults were injected with *cyk-4* dsRNA and the injected animals were analyzed 30 hours after injection (B). The gonad of an uninjected worm is shown for comparison (A). Wild-type embryos (C) and 5 embryos from *cyk-4(RNAi)* injected animals (D) were fixed and stained for tubulin. 10  $\mu$ m scale bars.

RNAi of the predicted open reading frame K08E3.6 generated multinucleate embryos which exhibited a similar phenotype to that of the *cyk-4* mutant, 10 including loss of the central spindle (figure 4 D) and incomplete cytokinesis (not shown). Interestingly, the gonads of *cyk-4(RNAi)* animals become disorganized 30 hours post-injection (figure 4 B) and irregularly sized embryos are produced, suggesting that CYK-4 acts 15 not only during embryonic and zygotic development, but also in the female germline.

#### Example 3

##### Biochemical activity of the Cyk-4 GAP domain

20 The presence of a Rho-family GAP domain suggests that CYK-4 may regulate one or more GTPases of Rho branch of the GTPase superfamily. To determine whether CYK-4 is active as a GAP and whether its GAP activity is restricted to particular members of the Rho subfamily, 25 a recombinant fusion protein containing GST and the CYK-4 GAP domain was prepared and GTP hydrolysis assays were performed with recombinant *C. elegans* Rho, Rac, and Cdc42. The GAP domain of CYK-4 promotes GTP hydrolysis by all three tested GTPases. Figure 5 shows

that CYK-4 enhances GTP hydrolysis by Rho, Rac, and Cdc42. The GTPases were preloaded with  $^{32}\text{P}$ - $\alpha$ -GTP and then GST-CYK-4-GAP or GST was added at the indicated concentration. Samples were taken at two minute 5 intervals, the labeled nucleotide was resolved by thin layer chromatography, the fraction of GTP and GDP was quantitated. This graph shows the fraction of GTP hydrolyzed at the 2 minute time point as a function of CYK-4 concentration. These data are representative of 10 at least three independent experiments.

However kinetic differences were observed. At the conditions used in the assays it was found that the CYK-4 GAP domain is more active towards Rac and Cdc42 than towards Rho. The human ortholog has a similar 15 activity profile *in vitro* (Toure et al., 1998). However, since CYK-4 has activity towards all three GTPases, these data are not sufficient to determine the *in vivo* target(s) of the CYK-4 GAP domain.

Next RNAi was used to determine which, if any, of the 20 Rho family GTPases are required for cytokinesis in the *C. elegans* embryo. RNAi experiments were performed with RhoA, Rac1, Cdc42 and three additional GTPases found in the genome that fall into the Rho subfamily. Approximately 90% of *Rho*(RNAi) embryos exhibit 25 cytokinesis defects in the first and/or second cell cycle (table 2 and figure 6).

Figure 6 shows that *rho*(RNAi) causes cytokinesis defects and *cdc42*(RNAi) causes defects in spindle positioning. Young adults were injected with the 30 indicated dsRNAs (see table 2) and the embryos produced

by the injected worms were analyzed by time lapse nomarski microscopy. Images from a wild-type (A), *rho*(*RNAi*) (B) and a *cdc42*(*RNAi*) (C) embryo are shown. 10  $\mu$ m scale bars.

5 In most embryos, furrow ingression was inhibited. Interestingly, central spindles assemble in *RhoA*(*RNAi*) embryos (data not shown). In contrast, 88% of *Cdc42*(*RNAi*) embryos complete cytokinesis normally. However, a distinct defect in the early embryo is

10 observed in 54% of *Cdc42*(*RNAi*) embryos; defects in spindle positioning are observed in P0 and/or P1. A minority (12%) of *Cdc42*(*RNAi*) embryos fail to initiate cytokinesis; in most cases, these embryos appear osmotically swollen even when provided with osmotic

15 support. *Rac*(*RNAi*) embryos hatch with high efficiency and did not exhibit a detectable phenotype in the early embryo. RNA interference experiments with the additional GTPases either alone or in combinations did not reveal any additional defects in the early embryo.

20 Thus *RhoA* is the only member of the *Rho* family that is clearly required for cytokinesis and is therefore likely to be the critical target for the CYK-4 GAP domain.

25 Example 4

The subcellular localization of Cyk-4

Next the subcellular localization of CYK-4 protein was determined. CYK-4 localization is cell cycle dependent

Figure 7 shows that CYK-4 localizes to the central spindle and division remnants. (A-F) Wild-type embryos were fixed and stained for CYK-4 (green), tubulin (red) and DNA (blue). The localization of CYK-4 to the 5 central spindle (arrow) in a one cell embryo (D) and a two cell embryo (F) is shown. The localization of CYK-4 to the division remnant from the polar body (A), and between the AB and P1 blastomeres is indicated with arrows. (E). (G) An embryo from a line expressing 10 CYK-4:GFP is stained with anti-GFP antibodies. The same structures are seen as with CYK-4 antibodies (arrow). (H) The intrinsic fluorescence of the gonad of a worm expressing CYK-4:GFP. CYK-4 is seen at the incomplete membranes of the syncytial gonad (arrow) and 15 in oocyte nuclei. 10  $\mu\text{m}$  scale bars.

In interphase cells, CYK-4 is present in the cytoplasm and slightly concentrated in the nucleus. CYK-4 is also highly concentrated in a spot at the anterior of the embryo, DNA labeling reveals that this localization 20 corresponds to the site of polar body extrusion. As embryos enter mitosis, CYK-4 protein concentrates around the mitotic spindle. In early anaphase, CYK-4 concentrates to the central spindle. As the cleavage furrow ingresses, CYK-4 becomes highly concentrated on 25 the central spindle into a structure that often appears ring shaped (not shown). Upon completion of cytokinesis, CYK-4 staining persists at division remnants. CYK-4 organized in ring-like structures averaging 1.2  $\mu\text{m}$  in diameter are occasionally observed 30 in the cytoplasm (not shown).

To determine if CYK-4 localizes to the central spindle prior to the onset of cleavage furrow ingression , the dynamics of CYK-4 localization in live embryos was investigated. To accomplish this goal a transgenic 5 line expressing a CYK-4:GFP fusion was generated and its localization followed by time lapse microscopy. The CYK-4:GFP fusion is partially functional since *cyk-4 xsEx1[cyk-4:GFP]* animals are viable and fertile at 25°C whereas the parental *cyk-4* strain is inviable 10 at 25°C. However, the fusion construct does not fully rescue the mutation, since about 40% of embryos produced by this line fail to hatch (table 1).

Low light level fluorescence microscopy was used to visualize CYK-4:GFP in living embryos. Embryos were 15 imaged using a multi-mode imaging system whereby a series of z-sections and a nomarski image were recorded every 10 seconds. The fluorescent images from each time point were projected to form a single image.

Figure 8 shows the time lapse analysis of CYK-4:GFP. 20 An embryo from a line expressing CYK-4:GFP was imaged using low light level microscopy. The central spindle localization of CYK-4 is observed prior to furrow ingression. 10  $\mu\text{m}$  scale bars.

These recordings reveal that CYK-4 accumulates on the 25 central spindle prior to the initiation of furrowing (figure 8; 3:40). The CYK-4 that localizes to the central spindle becomes compressed into a bright spot which persists at the division remnant. The remnant persists for several cell cycles although instances 30 were observed whereby the remnant (sometimes from the

polar body) detaches from the cortex and is observed as a discrete spot in the cytoplasm. This detachment of CYK-4 from division remnants likely accounts for the CYK-4 rings seen in fixed specimens. It is concluded  
5 that CYK-4 localization on the central spindle precedes furrow ingressions.

#### Example 5

Cyk-4 and zen-4/CeMklp1 are functionally interdependent  
10 There are remarkable similarities between CYK-4 and the kinesin-like protein ZEN-4/CeMKLP1 (Powers et al., 1998; Raich et al., 1998). zen-4 mutant embryos also initiate, but fail to complete cytokinesis. They also fail to assemble a robust central spindle in early  
15 anaphase. Furthermore, ZEN-4 localizes to the central spindle and persists at division remnants after completion of cytokinesis. To test whether these proteins functionally interact, it was first assessed whether CYK-4 and ZEN-4/CeMKLP1 co-localize.  
20 Embryos expressing CYK-4:GFP were fixed and CYK-4 and ZEN-4 were localized simultaneously (using an anti-GFP antibody to detect CYK-4:GFP). The two proteins co-localize both on central spindle structures and on division remnants (Figure 9A-C). It was next  
25 investigated if ZEN-4 localization requires functional CYK-4 protein. ZEN-4 staining of *cyk-4* mutant embryos reveals that ZEN-4 localization to the central spindle is absent (figure 9E), although staining of some microtubule bundles in the spindle midzone could be

detected using an AIR-2 antibody (figure 9G). Thus, recruitment of ZEN-4 to the central spindle is CYK-4 dependent.

Figure 9 shows an embryo expressing CYK-4:GFP stained for GFP (A), ZEN-4/CeMKLP1 (B) and the merged image (C). CYK-4 and ZEN-4/CeMKLP1 colocalize at division remnants (arrow) and a central spindle (arrowheads) structures. ZEN-4 localization to the central spindle is CYK-4 dependent (arrow). Wild-type (D) and *cyk-4(t1689ts)* embryos (E) were fixed and stained for ZEN-4/CeMKLP1 (green), tubulin (red) and DNA (blue). AIR-2 localization to the central spindle is CYK-4 independent (arrow). Wild-type (F) and *cyk-4(t1689ts)* embryos (G) were fixed and stained for AIR-2 (green), tubulin (red) and DNA (blue). CYK-4 maintenance to division remnants (arrowheads) is ZEN-4/CeMKLP1 dependent. *zen-4(or153ts)* worms were maintained at 16°C and either fixed immediately (H) or shifted to 25°C for 18 minutes (I). Embryos were fixed and stained for CYK-4 (green), tubulin (red) and DNA (blue). 10  $\mu$ m scale bars.

Next it was tested whether maintenance of ZEN-4 at division remnants requires functional CYK-4. *cyk-4* mutant embryos grown at 16°C were shifted to 25°C for 15 minutes prior to fixation and staining with anti-ZEN-4 antibodies. The number of cells and number of division remnants labeled with the anti-ZEN-4 antibody were counted. Embryos maintained at the permissive temperature had a large number of ZEN-4 staining division remnants (table 3) whereas the embryos shifted to the non-permissive temperature lacked defined

staining of division remnants. Thus both recruitment of ZEN-4 to the central spindle and its maintenance at division remnants is CYK-4 dependent. The reverse experiment was conducted with a temperature sensitive 5 allele of *zen-4* (*or153ts*). In this case CYK-4 staining at division remnants was observed in *zen-4* mutant embryos at the permissive temperature but this staining disappeared upon a brief shift to the non-permissive temperature (figure 9H,I and table 3). Thus maintenance 10 of CYK-4 at division remnants is ZEN-4 dependent. It is concluded that CYK-4 and ZEN-4/CeMKLP1 colocalize and that the two proteins are interdependent for their 15 localization.

Next, it was determined if embryos carrying mutations 20 in both *cyk-4* and *zen-4* are distinguishable from the single mutants. Two strains were built, one strain was homozygous for *zen-4* (*or153ts*) and heterozygous for *cyk-4* (*t1689ts*) and the second strain was homozygous for *cyk-4* (*t1689ts*) and heterozygous for *zen-4* (*or153ts*). Both strains were viable at 16°C, but they failed to 25 produce doubly homozygous larvae. It was found that worms of genotype *unc-32* (*e189*) *cyk-4* (*t1689ts*) / *qC1* III, *zen-4* (*or153ts*) laid a fraction of embryos that arrested during embryonic development, typically before the comma stage. Thus *cyk-4* (*t1689ts*) and 30 *zen-4* (*or153ts*) are synthetically lethal.

In the subsequent Examples, the following Materials and Methods were used:

## i) Worm Strains and Alleles

The following alleles were used: N2(Bristol), CB4856, *cyk-4(t1689ts)*, *unc-64*, *bli-6(sc16)*, *unc-24(e138)*, 5 *unc-44(e1260)*, *lag-1(q385)*, and *mIs11 IV*. Some strains were obtained from the *C. elegans* Genetics Center.

ii) Isolation and mapping of *cyk-4* suppressor alleles

Suppressors of the *cyk-4(t1689ts)* mutation were obtained by mutagenizing *cyk-4(t1689ts)* animals with 10 30-40 mM EMS or 0.5 mM ENU. P0 animals were allowed to self fertilize for two generations at the permissive temperature. When F2 animals reached early adulthood, the population was shifted 20°C and fertile animals were selected. Approximately 110,000 F1 genomes were 15 screened and 18 suppressor mutations, all but 2 being unambiguously independent, were isolated. While all of the suppressor strains were viable and fertile at 20°C, none were able to grow at 25°C, suggesting that none of them precisely reverted the original mutation. Several 20 intragenic suppressor mutations were identified. The ZEN-4 coding region of the remaining mutants were sequenced and 7 strains were found to contain substitutions within the CYK-4 binding region of ZEN-4 and one contained a substitution in the catalytic 25 domain. One suppressor strain, *xs82*, was characterized in detail. Suppressor activity was mapped using single nucleotide polymorphisms (SNPs) to the central region of chromosome IV (see methods). The ZEN-4 coding region was sequenced in this strain and a point mutation was

found that causes a substitution of glutamic acid for a lysine at position 502 (fig 15A).

iii) Cell Culture

HeLa cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin.

iv) Antibodies

Rabbit CYK-4 specific and ZEN-4 specific antisera were reported previously (Jantsch-Plunger et al., 2000). CYK-4 and ZEN-4 antibodies were affinity-purified with His6-CYK-4 (407-613) or His6-ZEN-4 (578-775) immobilized on NHS-Sepharose (Pharmacia).

MKLP-1 specific antisera and HsCYK-4 specific antisera were produced in rabbits and mice (Gramsch Laboratories, Schwabhausen, Germany) using C-terminal peptides (QLGPGYQHHAQPKRKKP and SKSKSATNLGRQGNFFASPMLK, respectively) conjugated to keyhole limpet hemocyanin as immunogens. Rabbit antibodies were affinity purified using peptides immobilized on Poros epoxide resin.

v) Plasmids

A cDNA clone for ZEN-4 in pBluescript (yk391b3) which lacks 13 nts at 5' of the coding region which were introduced by PCR. The pCBD-TEV vector was constructed by inserting the sequences encoding the chitin-binding domain (CBD) and a TEV protease site into pET28a

(Novagen). ZEN-4 fragments were amplified by PCR and cloned into pCBD-TEV.

vi) In Vitro Binding Assay

Full length and fragments of CYK-4 and ZEN-4 were  
5 expressed by in vitro transcription and translation  
system with reticulocyte lysates using the TNT® Coupled  
Reticulocyte Lysate Systems (Promega) or the  
PROTEINscript™ II (Ambion) kits, typically in 20  $\mu$ l  
reactions. Full length proteins without tag were  
10 expressed from the T3 promoter of pBluescript SK(-).  
Fragments of CYK-4 and ZEN-4 tagged with chitin-binding  
domain (CBD) at N-terminus were expressed from the T7  
promoter of pET-CBD. In some experiments (Fig. 12C,  
13C and 16), CYK-4 and ZEN-4 were co-expressed in the  
15 same reactions. In other experiments, they were  
separately expressed and mixed. Following incubation  
at 20°C for 30 min in 100  $\mu$ l buffer A (20 mM Hepes,  
150 mM NaCl, 2 mM MgCl<sub>2</sub>, 10 mM EDTA, 1 mM DTT, 1 mM  
PMSF, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin, 10  $\mu$ g/ml  
20 chymostatin) with 0.5% (w/v) Triton X-100, ZEN-4 or  
CYK-4 proteins were immunoprecipitated with specific  
antibodies, or affinity-purified by chitin beads. For  
immunoprecipitation, 0.5  $\mu$ g antibody was added to the  
reactions and incubated on ice for 1 hr, followed by  
25 incubation with 5  $\mu$ l protein A-Sepharose beads at 4°C  
for 1h. For CBD-affinity purification, 5  $\mu$ l of chitin  
beads (New England Biolabs) were added. In both cases,  
the beads were washed three times with buffer A  
containing 0.5% (w/v) Triton X-100. Proteins bound to  
30 the beads were analyzed by SDS-PAGE followed by  
autoradiography with PhosphoImager.

## vii) Preparation of Cell Lysates

C. elegans embryos were prepared by bleaching a synchronous culture of adult worms grown on "egg plates" as described (Lewis and Fleming, 1995). The 5 embryos were washed with buffer A without Triton-X 100 and frozen with liquid nitrogen and kept at -80°C until use. Embryos were crushed by grinding in a mortar and pestle in liquid nitrogen.

HeLa cells were synchronised by treatment with 0.1 10 µg/ml nocodazole for 16 h. Cells were washed twice with cold PBS, frozen by liquid nitrogen and kept -80°C until use. Cells were lysed and thawed by suspending into 10 volumes of buffer A with 0.5% (w/v) Triton X-100.

## 15 viii) Preparation of Microtubule-binding Fraction

Frozen pellet (0.5 ml) of nocodazole arrested M-phase cells was thawed and lysed in 5 ml ice-cold BRB80 (80 mM K-Pipes, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, pH 6.8) with 0.1% (w/v) Triton X-100. The lysate was clarified by 20 centrifuging at 10,000 x g for 15 min. The resulting supernatant was centrifuged at 25,000 x g for 20 min at 4°C in S100AT6 rotor (Hitachi). Microtubules polymerized with glycerol and taxol was added to the supernatant. After incubating at 20°C for 20 min, 25 microtubules were pelleted by centrifuging at 25,000 x g for 20 min at 20°C. The bound proteins were released from microtubules by incubation in 0.5 M NaCl, 5 mM ATP in BRB80 at 20°C for 20 min, followed by centrifugation at 25,000 x g for 20 min at 20°C.

## ix) Mass Spectrometry analysis

## Hydrodynamics

Sedimentation coefficient was estimated by ultracentrifugation through 2 ml linear gradient of 5 to 20% (w/v) sucrose in buffer A without Triton X-100 using S55S rotor (Hitachi). 150  $\mu$ l cell lysate or microtubule binding fraction was applied. Chicken ovalbumin (3.4 S), bovine gamma globulin (7.1 S), bovine catalase (11 S) and bovine thyroglobulin (19 S) were used as standards. The diffusion coefficient was estimated by gel filtration using Superdex 200 column (30 ml) in FPLC system (Pharmacia).

## Immunoprecipitation and Western Blotting

Lysates (500  $\mu$ l) were precleared with 50  $\mu$ l protein A-Sepharose beads. For immunoprecipitation from worm embryos, 1  $\mu$ g affinity-purified antibody was added to the lysate. After incubation on ice for 1 hr, immunocomplex was recovered by incubation with 5  $\mu$ l protein A-Sepharose (Pharmacia) at 4°C for 1 hr. For immunoprecipitation from HeLa cells, antibodies were covalently immobilized on protein A-beads (1  $\mu$ g per 1  $\mu$ l beads) with dimethyl pimelimidate. The precleared lysate was incubated antibody-beads at 4°C for 1 to 4 h. The beads were washed briefly with buffer A plus 0.5 % Triton X-100 three times.

For western blotting, samples were run on 7.5% SDS-PAGE gel and electrotransferred to a nitrocellulose membrane (HiBond ECL, Amersham).

## x) Immunolocalization

Immunolocalization studies in worm embryos were performed as previously described ref. In brief, gravid hermaphrodites were placed on aminopropyl-silane 5 treated slides, a coverslip was added, and sufficient pressure to extrude the embryos was applied (Jantsch-Plunger et al., 2000). The slide was placed into liquid nitrogen. The coverslip was removed while the sample was still frozen, the preparation was fixed with -20°C 10 methanol, and antibody staining was performed according to standard procedures. Immunolocalization studies using HeLa cells were performed according to standard procedures following fixation in -20 °C methanol.

## 15 Example 6

Central spindle assembly is independent of RhoA

The process of central spindle assembly is dependent on the RhoGAP protein CYK-4. In the previous Examples, it was found that RhoA is essential for cytokinesis while 20 Rac and Cdc42 are not (Jantsch-Plunger et al., 2000). Since RhoA is required for cytokinesis, it was tested if this GTPases is also required for central spindle assembly. To inactivate RhoA worms were grown on bacteria expressing dsRNA from the coding region of the 25 gene (Timmons and Fire, 1998). Embryos were fixed and immunoflorescence-stained for CYK-4, microtubules and DNA with anti-CYK-4 antibody, anti- $\alpha$ -tubulin antibody and Hoechst 33342). In both control embryos and

*rhoA*(RNAi) embryos, central spindles formed and were prominently labeled with anti-CYK-4 antibodies.

Although RhoA dsRNAi causes a fully penetrant inhibition of cytokinesis, central spindle assembly 5 occurs normally, and CYK-4 localizes to the central spindle (figure 11). Thus the requirement for RhoA in cytokinesis can not be accounted for by a requirement for RhoA in central spindle assembly. These data indicate that the requirement for CYK-4 in central 10 spindle assembly is independent of its ability to regulate RhoA.

#### Example 7

CYK-4 and ZEN-4 form a complex in vivo and in vitro 15 Since central spindle assembly and cytokinesis requires both CYK-4 and ZEN-4 and these two proteins are interdependent for their proper localization, it is possible that these factors exist in a stable biochemical complex. In order to test this 20 possibility, immunoprecipitation experiments were performed using extracts prepared from early *C. elegans* embryos. When embryo extracts were immunoprecipitated using anti-CYK-4 antibodies, it was found that significant amount of ZEN-4 co-immunoprecipitated 25 suggesting the existence, in vivo, of a stable complex containing CYK-4 and ZEN-4 (fig. 12A).

To determine whether CYK-4 and ZEN-4 are competent to interact in the absence of other nematode proteins, the two proteins were produced by in vitro translation and

subjected to immunoprecipitation. When CYK-4 and ZEN-4 (fused to the chitin binding domain (CBD)) were translated in vitro and subsequently mixed, both proteins could be recovered in high yield by chitin beads (fig. 12B). The co-precipitation of CYK-4 was dependent on ZEN-4; CYK-4 was not recovered on chitin beads when ZEN-4 was substituted by CBD alone. Moreover, when luciferase was substituted for CYK-4, it did not co-precipitate with ZEN-4. The association between CYK-4 and ZEN-4 could also be detected when anti-CYK-4 antibodies were used to retrieve the complex (fig. 12C).

Figure 12 shows that CYK-4 and ZEN-4 bind to each other in vivo (A) and in vitro (B and C). (A) CYK-4 and ZEN-4 were immunoprecipitated from worm embryo extracts with anti-CYK-4 antibody, anti-ZEN-4 antibody, or with non specific rabbit IgG as a control. The immunoprecipitates were resolved on SDS-PAGE gels followed by western blotting with an anti-ZEN-4 antibody. Mock immunoprecipitation without extract was included to control for cross reactivity with the antibodies. ZEN-4 specifically co-immunoprecipitated with CYK-4. (B) <sup>35</sup>S-labeled CYK-4 (or luciferase, LUC) and chitin-binding domain (CBD)-tagged ZEN-4 (or CBD alone) were separately expressed by in vitro translation (left panel). Translation reactions were mixed and incubated in the indicated combinations and CBD-ZEN-4 or CBD alone were recovered with chitin beads (right panel). In this and all gels in this paper the input lanes contain the same amount of translation product as was added to the beads used for precipitation. The precipitates were resolved on

SDS-PAGE gels and the labeled products detected using a phosphoimager. CYK-4 co-purified with CBD-ZEN-4 while the luciferase control did not. (C) CYK-4 (or luciferase) and CBD-ZEN-4 (or CBD alone) were 5 co-expressed as <sup>35</sup>S-labeled proteins in the indicated combinations (left panel). CYK-4 was immunoprecipitated with an anti-CYK-4 antibody (right panel). ZEN-4 co-immunoprecipitated with CYK-4.

10 Example 8

Delineation of the region of CYK-4 necessary to bind to ZEN-4

Since the binding between CYK-4 and ZEN-4 could be easily reconstituted using in vitro translated 15 proteins, this simple assay was used to dissect the regions of the two proteins that mediate this interaction. The experiment first concentrated on CYK-4. The N-terminal 30 amino acids of CYK-4 are poorly conserved and are followed by a 90 amino acid region 20 predicted to form a coiled-coil (see schematic fig. 13D). The C-terminal 250 amino acids contains a RhoGAP domain that is active in vitro against RhoA, Rac, and Cdc42 (Jantsch-Plunger et al., 2000). The RhoGAP domain is preceded by a C1 domain, a cysteine-rich domain that 25 mediates interactions with diacylglycerol (Hurley and Meyer, 2001) other ref. Truncations of the C-terminus of CYK-4 revealed that the ZEN-4 binding region is contained within the N-terminal 232 amino acids (fig. 13A). Further truncations within this region 30 indicated that residues 1-120 (from the N-terminus to

the end of the coiled-coil region) are sufficient to bind efficiently to ZEN-4 (fig. 13B). Deletion of the N-terminal 35 amino acids of CYK-4 prevented the interaction with ZEN-4. A near full length version of CYK-4 lacking only the N-terminal 35 amino acids does not bind ZEN-4 (fig 13C). Thus the ability of CYK-4 to interact with ZEN-4 depends on the N-terminal 120 residues of CYK-4 (fig. 13D).

Figure 13 shows the results of the in vitro binding assay that was used to define the ZEN-4 binding region of CYK-4.

(A) Full-length ZEN-4 and C-terminal deletion fragments of CYK-4 (1-232, 1-373 and 1-468) were separately expressed as <sup>35</sup>S-labeled proteins by in vitro translation (left two panels). The reaction products were mixed and after incubation ZEN-4 was immunoprecipitated with an anti-ZEN-4 antibody (right panel). The three fragments of CYK-4 co-immunoprecipitated with ZEN-4. (B) Full length CYK-4 (1-681) and CYK-4 with N-terminal deletion (35-681) were expressed as <sup>35</sup>S-labeled proteins and mixed with unlabeled CBD-ZEN-4 (left panel). After incubation, CBD-ZEN-4 was affinity purified on chitin beads (right panel). CYK-4 (35-681) did not copurify with CBD-ZEN-4, but full length CYK-4 did. (C) CBD-CYK-4 fragments (1-232, 35-232, 121-232, 1-120 and 35-120) and full length ZEN-4 (or luciferase as a control) were co-expressed as <sup>35</sup>S-labeled proteins by in vitro translation (left panel). CBD-CYK-4 fragments were affinity purified (right panel). ZEN-4 copurified with CYK-4 (1-232) and (1-120), but not with CYK-4 (35-232),

(121-232) or (35-120). (D) A schematic summary of the CYK-4 derivatives tested. The ZEN-4 binding region was defined as residues 1-120 of CYK-4. This region contains a coiled coil region and a N-terminal

5 extension.

#### Example 9

##### Delineation of the ZEN-4 region required to bind CYK-4

Next, the in vitro assay was used to define the region  
10 of ZEN-4 that binds to the N-terminal binding of CYK-4. Initially the CYK-4 binding activity of full length ZEN-4 was compared to that of three C-terminal deletion fragments of ZEN-4: the N-terminal catalytic core alone (1-434), the catalytic core domain and the linker  
15 region (1-507), and a longer derivative that also includes the coiled coil domain (1-603). The full length protein bound CYK-4 with high affinity, as did the next longer fragment (1-603), but the two smaller fragments did not interact with CYK-4 (fig. 14A). Next  
20 it was examined whether the binding activity could be further localized within the central region of ZEN-4. A minimal binding domain consisting of 169 amino acids was defined (fig. 14B). This region appears to consist of two elements, the linker region and the coiled coil;  
25 neither of these individual elements had detectable binding activity on their own.

The interaction of CYK-4 with the neck linker/coiled coil region of ZEN-4 is particularly interesting in light of recent studies that indicate that in

conventional kinesin, the neck linker region is critical for transducing chemical energy into mechanical energy (Case et al., 2000; Rice et al., 1999) (see discussion).

5 Figure 14 shows the results of the in vitro binding assay that was used to show that the central region of ZEN-4 is necessary and sufficient to bind to CYK-4.

Full length ZEN-4 (1-775) and C-terminal deletion fragments of ZEN-4 (1-603, 1-507 and 1-434) were 10 <sup>35</sup>S-labeled by in vitro translation and incubated with unlabeled CYK-4 (or luciferase as a control) (left panel). CYK-4 was precipitated with anti-CYK-4 antibody (right panel). Full length ZEN-4 and ZEN-4 (1-603) co-precipitated with CYK-4, while shorter 15 fragments (1-507, 1-434) did not. (B) CBD-tagged fragments of ZEN-4 (1-603, 1-434, 435-603, 435-507 and 503-603) were expressed in an unlabeled form and incubated with <sup>35</sup>S-labeled CYK-4 (1-232) (left panel). ZEN-4 fragments were pulled down by affinity 20 chromatography using chitin beads (right panel). CYK-4 (1-232) copurified with ZEN-4 (1-603) and (435-603), but not with ZEN-4 (1-434), (435-507) or (503-603). (C) A schematic summary of the ZEN-4 derivatives 25 tested. ZEN-4 fragments containing residues (435-603) associate with CYK-4, while the fragments lacking this region did not. This region contains a "neck" region C-terminal to the kinesin catalytic core and a coiled coil region.

## Example 10

a) The product of *cyk-4(t1689ts)* does not interact with *ZEN-4*.

Although the amino terminal region of CYK-4 exhibits  
5 poor sequence conservation at the primary sequence  
level, earlier studies had indicated that this region  
is crucial for function since the mutant allele that  
was used to identify the *cyk-4* gene had a Ser to Leu  
substitution at position 15. Since this point mutation  
10 maps into the *ZEN-4* binding determinant and since, *in vivo*, this mutation seems to affect the ability of CYK-4 and *ZEN-4* to colocalize, it was tested whether this mutation also affects the ability of these two proteins to interact *in vitro*. The S15L mutation was introduced  
15 into the amino terminal fragment of CYK-4 and used for *ZEN-4* binding assays. Whereas an interaction between the N-terminal domain of CYK-4 and the central region of *ZEN-4* can be readily detected, the S15L mutant does not interact with *ZEN-4* (fig. 15B). This experiment  
20 indicates that the primary cause for the defect in *cyk-4(t1689ts)* is a defect in the interaction with *ZEN-4*.

b) *In vivo*, *ZEN-4* is in a stoichiometric complex with Cyk-4

To gain further insight into the molecular architecture  
25 of the CYK-4/*ZEN-4* complex and to determine whether a  
the majority of *ZEN-4* is in a complex with CYK-4 *in vivo*, sucrose density gradient centrifugation  
experiments were performed with extracts prepared from  
30 *C. elegans* embryos. When wild-type extracts are prepared in the presence of 0.6 M NaCl, *ZEN-4* migrates

as a symmetrical 9 S peak (fig. 15C). CYK-4 migrates at an identical S-value (data not shown). To determine whether the migration of ZEN-4 on sucrose gradients was dependent on its association with CYK-4, advantage was 5 taken of the finding that the *cyk-4(t1689)* allele is specifically defective in its ability to bind to ZEN-4. When extracts were prepared from *cyk-4* mutant embryos and subjected to sucrose density centrifugation, ZEN-4 migrates as a symmetrical peak with a sedimentation 10 value of 6 S, significantly less dense than ZEN-4 sediments in wild type extracts (fig. 15C). These data indicate that, *in vivo*, the majority of ZEN-4 is in a complex with CYK-4.

c) *In vivo* evidence for the CYK-4/ZEN-4 interaction 15 domains

To gain further insight into the mechanism of CYK-4 function *in vivo*, it was sought to identify extragenic suppressors of the *cyk-4(t1689ts)* mutation. Homozygous *cyk-4(t1689ts)* animals were mutagenized and allowed to 20 self fertilize for two generations at the permissive temperature. When the F2 animals reached early adulthood, the temperature was shifted to 20°C to impose a selection for fertile animals carrying presumptive suppressor mutations. By screening through 25 approximately 100,000 F1 genomes, 18 suppressor mutations were identified (see methods). Intragenic suppressors were identified by sequencing of the *cyk-4* locus PCR amplified from suppressor strains. Six suppressor strains contain additional point mutations 30 in the N-terminus of CYK-4; all of the mutations code

for amino acid substitutions within the biochemically defined ZEN-4 binding region.

In addition to the mutations found within the *cyk-4* gene itself, extragenic suppressor mutations were also 5 identified. One suppressor strain that does not contain any mutations in the *cyk-4* gene was characterized in more detail. The suppressor activity was mapped using single nucleotide polymorphisms (SNPs) to the central region of chromosome IV (see methods). 10 Since the *zen-4* gene maps to this region, the coding region of the *zen-4* gene was sequenced. This strain, *xs82*, was found to contain a point mutation that causes a substitution of glutamic acid for a lysine at position 502 (fig. 15A). Importantly, this substitution 15 maps directly to the region of ZEN-4 that was found to mediate the interaction with CYK-4 in vitro. To confirm that the substitution in *zen-4* (*xs82*) is responsible for the suppressing activity, the position of the suppressor was mapped to less than 1 cM 20 surrounding the *zen-4* locus. This mapping data combined with the fact that the sequence polymorphism maps to the CYK-4 binding region suggests strongly that these point mutations are responsible for the suppressing activity. 25 To determine whether the substitution, E502K, has any phenotype on its own, in the absence of the *cyk-4* (*t1689*) allele, *cyk-4* (*t1689*) was crossed out and worms homozygous for *zen-4* (*xs82*) were identified. These worms grew normally at 16°C, 20°C, and 25°C.

d) ZEN-4 (xs82) partially restores binding to CYK-4 in vitro

As mentioned above, CYK-4 S15L is unable to interact with ZEN-4 in the in vitro binding assay. If the 5 immediate consequence of the S15L substitution in the N-terminus of CYK-4 is defective ZEN-4 binding, then an extragenic suppressor mutation in *zen-4* that rescues *cyk-4(t1689)* might be expected to restore binding to CYK-4 S15L. To test this possibility a N terminal 10 fragment of CYK-4 carrying the S15L point mutation was translated and this product was mixed with a C terminal fragment of ZEN-4 carrying either the wild-type glutamic acid at position 502 or the lysine substitute allele. The wild-type CYK-4 fragment bound equally well 15 to wild-type and the E502K derivative of ZEN-4. As discussed above the CYK-4 S15L is unable to bind to wild-type ZEN-4. However, CYK-4 S15L was able to bind to ZEN-4 E502K, though this interaction was weaker than that seen with the wild-type proteins (fig. 15A). These 20 data, together with the genetic suppression, prove that the interaction between the N-terminus of CYK-4 and the central region of ZEN-4 is crucial for its function in vivo.

Figure 15 shows that a complex of CYK-4 and ZEN-4 is 25 required for function in vivo

(A) A screen for suppressors of *cyk-4(t1689ts)* identified a point mutation of *zen-4(xs82)*. This allele has a G to A point mutation, resulting in substitution of glutamate at residue 502 to lysine. 30 (B) The E502K mutation partially restores binding to

CYK-4 S15L.CYK-4 fragments (1-232, 1-232 with a point mutation S15L, 35-232) were expressed as  $^{35}\text{S}$ -labeled protein and incubated with unlabeled ZEN-4 fragments (435-775, 435-775 with the E502K mutation, 609-775).

5 ZEN-4 fragments were precipitated with anti-ZEN-4 antibody. CYK-4 (1-232) co-precipitated with ZEN-4 (435-775) but not with ZEN-4 (609-775) as shown in Figure 13. The S15L mutation in CYK-4 abolishes its ability to bind to ZEN-4 (CYK-4 1-232 S15L and ZEN-4

10 435-775 wt). However, the suppressor mutation in ZEN-4 (E502K) partially recovered this interaction ((CYK-4 1-232 S15L and ZEN-4 435-775 E502K). The ZEN-4 E502K mutation does not affect the binding to wild-type CYK-4. (C) The majority of ZEN-4 is in a complex with

15 CYK-4 in vivo. Extracts were made from wild type (N2) embryos or *cyk-4(t1689ts)* embryos. Sedimentation of ZEN-4 was analyzed by sucrose density gradient centrifugation followed by western blotting. The samples were spiked with the indicated standards and

20 they ran identically in the two gradients. ZEN-4 from wild type embryos sediments at 8 S, whereas ZEN-4 from *cyk-4* mutant embryos sediments at 7S.

#### Example 11

25 CYK-4 and ZEN-4 self-associate

Thus far it has been established that central spindle assembly is dependent on an interaction between CYK-4 and ZEN-4 and have defined the regions of both proteins that are required for this interaction. Interestingly, 30 both binding domains contain coiled coil regions; these

regions are required for binding activity. Since coiled coils are often dimerization motifs, and since kinesin motors are often composed of two motor domains tethered together through a coiled coil, it was 5 examined whether CYK-4 and ZEN-4 are individually able self-associate.

To assess if CYK-4 self associates, full length CYK-4 and the CYK-4 derivative 1-232 were co-expressed. As a control, the full length protein was substituted by a truncated version lacking the N-terminal 120 amino acids. The longer CYK-4 fragments were immunoprecipitated with an antibody directed against the C-terminus of CYK-4 and the co-immunoprecipitation of the short N-terminal fragment was assessed. The N-terminal fragment bound to full length CYK-4 but not to CYK-4 lacking the N-terminal domain (figure 16A). These data demonstrate that CYK-4 self associates. This association is likely mediated by the coiled-coil domain.

The capacity of ZEN-4 to self-associate was examined using a similar strategy. Full length ZEN-4 was co-expressed with affinity-tagged deletion derivatives of ZEN-4. Neither the N-terminal region (1-507) nor the C-terminal region (604-775) of ZEN-4 bound appreciably to full length ZEN-4, but two internal fragments of ZEN-4 (503-775; 435-603) did bind to full length ZEN-4 (figure 16B). The region common to these two fragments consists of residues 503-603 which is the region predicted to form a coiled coil domain.

The ability of CYK-4 and ZEN-4 to self associate in vitro raise the possibility that in vivo, the CYK-4/ZEN-4 complex may contain, at a minimum, two molecules each of CYK-4 and ZEN-4.

5 Figure 16 shows the self-association of both CYK-4 and ZEN-4

(A) CYK-4 (1-232), which does not contain the epitope recognized by the anti-CYK-4 antibody, was co-expressed as a <sup>35</sup>S-labeled protein with CYK-4 (1-681) or 10 CYK-4 (121-681), which do contain the epitope for this antibody (left panel). CYK-4 (1-681) and (121-681) were immunoprecipitated. CYK-4 (1-232) co-immunoprecipitated with CYK-4 (1-681), but not with CYK-4 (121-681). (B) CBD-tagged fragments of ZEN-4 15 (1-507, 503-775, 435-603 or 604-775) were co-expressed with untagged ZEN-4 (1-775) (left panel). CBD-tagged fragments were purified by affinity chromatography with chitin beads (right panel). Untagged ZEN-4 (1-775) copurified with CBD-ZEN-4 (503-775) and (435-603), but 20 not with ZEN-4 (1-507) or (604-775). The low extent of labelling of 604-775 as compared to 435-603 is attributable to low cysteine and methionine content of the C-terminal fragment.

25 Example 12

The human orthologs of CYK-4 and ZEN-4, HsCYK-4 and MKLP1, interact in vivo and colocalize.

In *C. elegans*, CYK-4 and ZEN-4 associate in vivo and in vitro and this complex is important for central spindle

assembly and cytokinesis. To extend these findings it was evaluated whether mammalian cells contain a stable complex containing the human orthologs of these proteins, HsCYK-4/MgcRacGAP (Hirose et al., 2001; Toure 5 et al., 1998; Wooltorton et al., 1999) and MKLP1, respectively. Extracts were prepared from mitotic HeLa cells and antibodies directed against HsCYK-4 or MKLP1 were used for immunoprecipitation followed by western blotting. MKLP1 could be co-immunoprecipitated with 10 anti-HsCYK-4 antibodies and vice versa (fig. 17a). The immunoblots reveal that small amounts of HsCYK-4 and MKLP1 migrate with reduced mobility, likely due to phosphorylation.

To obtain evidence that these proteins play a similar 15 role in human cells as was shown previously for the nematode proteins, the localization of HsCYK-4 and MKLP1 was examined in cultured (HeLa) human cells. During all stages of the cell cycle the two proteins precisely co-localize (insets fig 17b). In early 20 anaphase, the two proteins localize to a discrete, central region of the central spindle. Three dimensional reconstruction of optically sectioned anaphase cells reveals that each bundle of microtubules in the central spindle is labeled at its distal end by 25 MKLP1 antibodies. During mid-to-late anaphase, the MKLP1 staining region is  $0.93 \pm 0.25 \mu\text{m}$  in length (n=56). This region largely corresponds to the region that appears deficient in tubulin staining, but this is a known artifact caused by epitope masking (Saxton and 30 McIntosh, 1987). The extent of staining is somewhat narrower than previous ultrastructural determinations of the extent of microtubule overlap ( $> 2 \mu\text{M}$ )

(Mastronarde et al., 1993). Later in cleavage, both proteins localize to a discrete, central portion of the midbody. During interphase, some cells contain HsCYK-4 and MKLP1 in the nucleus, whereas others are do not.

5 This is likely due to cell cycle regulated accumulation of these factors since, upon G2 arrest, HsCYK-4 and MKLP1 accumulate in the nucleus of all cells (data not shown). In addition, most cells contain brightly-staining, cortical ring-like structures. These

10 structures likely correspond to division remnants; previous time-lapse microscopy with GFP-tagged CYK-4 and ZEN-4 in nematode embryos revealed that the central spindle matures into the midbody that later develops into a persistent spot or ring in the cell cortex. Thus

15 there is a striking concordance between the biochemical properties and the subcellular localization of CYK-4/ZEN-4 in *C. elegans* embryos and HsCYK-4/MKLP1 in human cells. Since the names of the individual proteins vary from species to species, it is proposed to call

20 this complex "centralspindlin". It is reasonable to speculate that centralspindlin performs a similar function in these two systems.

To gain insight into the architecture of centralspindlin, the hydrodynamic properties of its

25 constituents were investigated. Lysates were prepared from mitotic HeLa cells and run on sucrose density gradients. Western blotting of the gradient fractions indicates that HsCYK-4 and MKLP1 precisely co-migrate on the gradients with an S-value of 8 S (fig. 17b).

30 This sedimentation behavior is similar to that of centralspindlin isolated from *C. elegans* embryos. The two proteins also co-migrated on a gel filtration

column with an apparent molecular weight of 800 kD (data not shown). Gel filtration chromatography can not accurately estimate native molecular mass of asymmetric particles, however, gel filtration data, combined with 5 S-value measurements, allows a more accurate estimation of the native molecular mass. When the experimental values of centralspindlin are combined in this way, the complex is estimated to have a native molecular weight of ~300 kDa.

10 To determine if equimolar amounts of HsCYK-4 and MKLP1 are present in centralspindlin and whether or not other proteins are also present, the complex was immunopurified from a mitotic MAP (microtubule-associated protein) fraction that was eluted with ATP

15 and 500 mM NaCl. Antibodies specific for HsCYK-4 and MKLP1 were used in parallel for the immunoprecipitation. Coomassie blue staining of both immunoprecipitates revealed two major bands and one minor band (fig. 17C). Mass spectrometry analysis

20 revealed that the slower migrating major band and the minor band are MKLP1 and the faster migrating major band is HsCYK-4. No additional bands reproducibly co precipitated with HsCYK-4 or MKLP1. Given that centralspindlin has a native molecular mass of 300 kD

25 and that it does not appear to contain any proteins in addition to HsCYK-4 (70 kD) and MKLP1 (100 kD) and given that both components are capable of self-association, it is concluded that centralspindlin is a tetramer containing two molecules each of HsCYK-4 and

30 MKLP1.

Figure 17 shows that there exists a complex of MKLP-1 and HsCYK-4 in HeLa cells

HsCYK-4/MgcRacGAP and MKLP-1, the human orthologs of CYK-4 and ZEN-4, respectively, exist as a complex in 5 mitotic HeLa cells. (A) HsCYK-4 and MKLP-1 were immunoprecipitated from HeLa cell lysate with specific rabbit antibodies (IP:HsCYK-4 or MKLP-1) and blotted with specific mouse antibodies (blot: HsCYK-4 or MKLP-1). HsCYK-4 co-immunoprecipitated with MKLP-1 and 10 vice versa. (B) HsCYK-4 and MKLP1 co-localize to the central spindle and the midbody. Hela cells were fixed and immunostained with anti-CYK-4, anti-MKLP1, anti-tubulin antibodies and DNA was stained with Hoechst. Shown are cells in interphase (a) and various stages of 15 cytokinesis (b-f). In main panels, staining of CYK-4 (green), microtubules (red) and DNA (blue) was shown. In inset, staining of CYK-4 (green) and MKLP1 (red) was shown. (C) HeLa cell lysates were analyzed by sucrose density gradient centrifugation followed by blotting 20 with anti-MKLP-1 antibody or anti-HsCYK-4 antibody. HsCYK-4 and MKLP-1 co-sediment at 8S. The lowest band reacting with the CYK-4 antibody is caused by proteolysis of the N-terminus. (D) MKLP-1 and HsCYK-4 complex was immunoprecipitated from a microtubule- 25 binding fraction (MTfr.) of mitotic HeLa cells with specific antibodies. Major 110 kDa and 75 kDa bands were identified by mass spectrometry as MKLP-1 and HsCYK-4, respectively. The minor band at 115 kDa also corresponds to MKLP1. Broad bands around 50 kDa and 30 bands near gel bottom are heavy (IgGH) and light (IgGL) chains of immunoglobulin leaked from antibody beads.

Table 1

genotype	stage at time of shift to 25°C	percent phenotype of viable surviving progeny	N
N2 (wild type)	embryos	98.8% wild-type	485
<i>cyk-4</i>	(not shifted)	70.2% fertile	198
<i>cyk-4</i>	embryos	1.5% sterile, highly Unc	324
<i>cyk-4</i>	L1 larvae	100.0% sterile, highly Unc	89
<i>cyk-4</i>	L2/L3	100.0% sterile, highly Unc	55
<i>cyk-4</i>	L4	100.0% fertile, lay dead embryos	52
<i>cyk-4, xsEx1 [cyk-4:GFP]</i>	embryos	59.4% fertile	350

5 Except for the N2 control, the complete genotype of the strain was *unc-32(e189)* *cyk-4(t1689ts)*. Gravid hermaphrodites were allowed to lay embryos for 2 hours at the permissive temperature. The adult was then removed and the number of embryos counted. After 24 hours at the indicated temperature the number of unhatched embryos was then counted.

Table 2

injected locus dsRNA		percent cytokinesis defective (N)	number of time lapse recording	number cytokinesi s defective	additional phenotypes
RhoA	Y51H4A.B	95% (175)	15	15 (100%)	
Rac-1	C09G12.8 B	Not emb. lethal	5	0	
Cdc42	R07G3.1	12% (74)	22	3 (14%)	symmetric 1st division (5/22); no rotation in P1(12/22); rotation of AB (2/22)
others	F22E12.2	Not emb. lethal			
	Y32F6B.3	Not emb. lethal			
	K03D3.9	Not emb. lethal			

Young adult hermaphrodites were injected with the  
 5 indicated dsRNAs and broods of laid embryos were scored  
 for embryonic lethality. dsRNAs that induced embryonic  
 lethality were further characterized by dissecting  
 embryos and scoring for multinucleate embryos and by  
 performing time lapse recordings and evaluating  
 10 cytokinesis, spindle orientation in the one-cell embryo  
 (symmetric or asymmetric first cleavage), and spindle  
 positioning in the P1 and AB blastomeres.

Table 3

genotype	temperature	total number of stained remnants	total number of cells	average number of remnants/ cell	number of embryos
N2 (wildtype)	25°C	110	137	0.80	16
<i>zen-4 (or198ts)</i>	16°C	61	83	0.73	10
<i>zen-4 (or198ts)</i>	25°C (18 min.)	11	147	0.07	24
<hr/>					
N2 (wildtype)	25°C	150	205	0.73	22
<i>cyk-4 (t1689ts)</i>	16°C	61	98	0.62	10
<i>cyk-4 (t1689ts)</i>	25°C (15 min.)	10	176	0.06	19

Wild-type and *zen-4 (or153ts)* embryos grown at the  
 5 indicated temperatures were fixed and stained for  
 Cyk-4. Similarly, Wild-type and *cyk-4 (t1689ts)* grown  
 at the indicated temperatures were fixed and stained  
 for Zen-4/CeMKlp1. The number of cells in each embryo and  
 the number of remnants staining with Cyk-4 or Zen-4  
 10 antibodies was counted.

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